FRAGILE X SPECTRUM DISORDERS

EDITED BY: Randi Jenssen Hagerman, David Hessl, Sarah Lippe and Francois Bolduc
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FRAGILE X SPECTRUM DISORDERS

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$GABA_A$ Alpha 2,3 Modulation Improves Select Phenotypes in a Mouse Model of Fragile X Syndrome

Tori L. Schaefer[1], Amy A. Ashworth[1], Durgesh Tiwari[2,3], Madison P. Tomasek[4], Emma V. Parkins[2], Angela R. White[2], Andrew Snider[2], Matthew H. Davenport[1], Lindsay M. Grainger[1], Robert A. Becker[1], Chandler K. Robinson[1], Rishav Mukherjee[2], Michael T. Williams[2,3], Jay R. Gibson[4], Kimberly M. Huber[4], Christina Gross[2,3†] and Craig A. Erickson[1,3*†]*

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Fragile X syndrome (FXS) is the most common cause of inherited intellectual disability. FXS is caused by functional loss of the Fragile X Protein (FXP), also known as Fragile X Mental Retardation Protein (FMRP). In humans and animal models, loss of FXP leads to sensory hypersensitivity, increased susceptibility to seizures and cortical hyperactivity. Several components of the GABAergic system, the major inhibitory system in the brain, are dysregulated in FXS, and thus modulation of GABAergic transmission was suggested and tested as a treatment strategy. However, so far, clinical trials using broad spectrum $GABA_A$ or $GABA_B$ receptor-specific agonists have not yielded broad improvement of FXS phenotypes in humans. Here, we tested a more selective strategy in *Fmr1* knockout (KO) mice using the experimental drug BAER-101, which is a selective $GABA_A$ α2/α3 agonist. Our results suggest that BAER-101 reduces hyperexcitability of cortical circuits, partially corrects increased frequency-specific baseline cortical EEG power, reduces susceptibility to audiogenic seizures and improves novel object memory. Other *Fmr1* KO-specific phenotypes were not improved by the drug, such as increased hippocampal dendritic spine density, open field activity and marble burying. Overall, this work shows that BAER-101 improves select phenotypes in *Fmr1* KO mice and encourages further studies into the efficacy of $GABA_A$-receptor subunit-selective agonists for the treatment of FXS.

Keywords: Fragile X syndrome, FXS, FMRP, GABA, EEG, audiogenic seizures, novel object recognition, UP states

INTRODUCTION

Fragile X syndrome (FXS) is the most common inherited form of intellectual disability and often associated with autism, anxiety, irritability, and attention deficit hyperactivity disorder (1). Although many potential small molecule candidates have been tested in clinical studies (2–4), there is currently no effective approved treatment of FXS.

FXS is caused by a trinucleotide (CGG) repeat expansion in the 5'UTR of the *FMR1* gene leading to its transcriptional silencing and loss of a single protein, the Fragile X Protein (FXP), also known as Fragile X Mental Retardation Protein (FMRP). FXP has many functions including, but not limited to, control of mRNA transport, translation, and stability, as well as binding to and regulating

ion channels, and DNA repair (5). *Fmr1* knockout (KO) mice, the most frequently used animal model for FXS, display many phenotypes reminiscent of the human condition. They show, for example, hyperactivity, altered social preference, and impaired cognition, and are widely used to investigate pathological mechanisms of FXS and to preclinically test novel therapeutic strategies (6).

A particularly prominent characteristic of loss of FXP in humans and animal models is excessive brain activity. Studies in mice demonstrate that loss of functional FXP leads to altered and increased neuronal and circuit excitability, manifest, for example, in impaired synaptic plasticity, elevated neocortical activity and increased susceptibility to audiogenic seizures (7). Notably, humans with FXS are more prone to develop epilepsy than the general population (8), exhibit sensory hypersensitivity (9), and recent EEG studies have shown increased gamma power activity, altered neuronal synchronization and impaired connectivity in the neocortex of individuals with FXS (10, 11). Together, these findings suggest that altered circuit excitability is a disease-relevant and translational phenotype in FXS.

While the underlying molecular mechanisms of increased neuronal activity are not fully understood, there is substantial evidence for altered inhibitory transmission in FXS caused by changes in the γ-aminobutyric acid (GABA)ergic system (12–14). There are two classes of GABA receptors, the heteropentameric anion-permeable GABA type A ($GABA_A$) receptor, which mediates fast inhibition (15), and the G-protein coupled GABA type B ($GABA_B$) receptor, which mediates slow inhibition (16). $GABA_A$ receptors consist of combinations of 19 different subunits (17). MRNA and protein of several of these receptor subunits are reduced in *Fmr1* KO mouse brain (18–21) and in humans (22). Moreover, expression of the rate-limiting GABA-synthesizing enzyme glutamic acid decarboxylase (GAD) is altered in *Fmr1* KO mice but the direction of the dysregulation is unclear and appears to depend on the brain region (18, 20, 23).

Based on these studies, GABA receptors were evaluated as potential therapeutic targets in FXS. These studies have mostly used receptor subunit-non-specific agonists of either $GABA_A$ or $GABA_B$ receptors (24, 25). Despite positive results using a $GABA_B$ agonist, arbaclofen (R-Baclofen, STX209) the active racemic enantiomer of baclofen, in the FXS mouse model (26), a large Phase III trial in individuals with FXS with this drug did not meet the defined endpoint criteria (24). Similarly, ganaxolone, a $GABA_A$ agonist did not lead to significant clinical improvements in humans (25) following positive preclinical reports (27). Interestingly, with both arbaclofen and ganaxolone, treatment-associated positive effects were noted in *post-hoc* subgroup analyses. This supports the therapeutic promise of targeting the GABAergic system in FXS, but also highlights the need to evaluate alternative, more selective GABA receptor modulators while working to build a priori justification of potentially targeting specific subgroups of persons with FXS with a specific GABA modulator.

The investigational drug BAER-101 (formerly known as AZD7325) is a selective GABA receptor modulator that activates the α2 and α3 subunits of the $GABA_A$ receptor. This specific pharmacologic profile leads to potential potent anxiolytic actions without the common sedative impact of non-selective $GABA_A$ agonists such as benzodiazepines (28). Here, we tested the effect of BAER-101 treatment on cortical circuit hyperexcitability, behavioral phenotypes, and memory in *Fmr1* KO mice. Our results suggest that low-dose BAER-101 may be beneficial to normalize circuit hyperexcitability and improve object recognition memory but did not improve anxiety- and repetitive behavior-related phenotypes in *Fmr1* KO mice. These studies encourage more detailed analyses in the mouse model and in humans to evaluate the potential benefits of BAER-101 and other $GABA_A$ subunit-selective agonists in FXS.

MATERIALS AND METHODS

Animals

Fmr1 KO mouse breeding colonies (29, 30) were established in the Rodent Barrier Facility at Cincinnati Children's Hospital Medical Center (CCHMC) and at University of Texas Southwestern (UTSW). All protocols were approved by the Institutional Animal Care and Use Committees at CCHMC or UTSW. Mice for this study were housed under 14/10 (CCHMC) or 12/12 (UTSW) light/dark cycle at controlled temperature and humidity. Test subjects were generated from the mating of female $Fmr1^{+/-}$ mice (30) with male WT mice on a C57BL/6J background. Male mice from these pairings were used as test subjects for juvenile audiogenic seizure tests and the adult behavior battery and extracellular signal-related kinase (ERK1/2) analysis. Mice used for neocortical slice recordings or EEG analysis and dendritic spine morphology were also generated by breeding female $Fmr1^{+/-}$ mice to male WT mice on a C57BL/6J background, but the *Fmr1* KO strain was originally obtained from the *Jackson Laboratories* (Bar Harbor, ME) (29). While the two *Fmr1* KO mouse strains used here are slightly different in how they were generated, they both do not express FXP. All mice were genotyped on postnatal day (P) 10–28 by ear clip and weaned on P28. Male *Fmr1* KO and WT littermates were used for experiments and group housed throughout testing (with dam and litter of 2–4 per cage). Audiogenic seizures and UP state analyses were performed at P21 because *Fmr1* KO mice in C57BL/6J background are only susceptible to audiogenic seizures during early development and UP states neocortical slices are most robust at this age (31). Behavior, EEG, ERK1/2, and dendritic spine analyses were done in adult (2–4 months old) mice. Electrode and transmitter implanting as well as many of the behavioral assays are difficult or impossible during juvenile periods.

Drug and Drug Dosing

BAER-101 (4-amino-8-(2-fluoro-6-methoxy-phenyl)-N-propyl-cinnoline-3-carboxamide hydrogen sulfate, formerly AZD7325) was obtained from AstraZeneca (Europe), and the vehicle Sulfobutylether-Beta-Cyclodextrin (SBECD) was supplied by AstraZeneca (US). SBECD is a pharmaceutical grade agent that is used as a solubilizing agent in drugs currently on the market in the US (Voriconazole). Mice were treated with either 3 mg/kg (high dose) or 1 mg/kg (low dose) of BAER-101 in 0.05% SBECD

or vehicle (0.05% SBECD) in a volume of 5 ml/kg (3BAER, 1BAER, or VEH, respectively). Drug doses were based on IC50 values and recommendations communicated by AstraZeneca and mimicked the parallel human trial design that likewise included a high and a low dose group. Juvenile mice (3 weeks) were dosed by oral gavage one time, 30 min prior to the start of the audiogenic seizure test. For mice in the adult behavior battery, dosing commenced 10 days prior to the start of behavior analysis with treatment continuing during behavior testing. The 22 gauge gavage needles were 1.5" needles with 1.25 mm ball (Cadence Science catalog # 7920). A gavage dosing volume of 5 ml/kg was used to reduce the amount of SBECD exposure. On behavior testing days, dosing was staggered such that a period of 0.5 h would separate the dose and start of behavior for each mouse. For EEG analyses, mice implanted with cortical EEG electrodes and wireless transmitters were treated daily with 1 mg/kg BAER-101 or vehicle for 10 days either by oral gavage as described above or by providing the drug in a single-serve portion of peanut butter (32). The mice were observed to ensure they consumed the entire peanut butter, which usually took 1–2 min but always under 5 min. We used this method successfully in the past to deliver drugs (32). We changed drug administration methods from oral gavage to single-serve peanut butter to avoid disturbing the transmitter and electrode implants. We did not detect an effect of dosing method on EEG measurements in the limited number of mice tested (*data not shown*).

Neocortical Slice Preparation and UP State Recordings

Slices from somatosensory barrel cortex (400 μm thick) were prepared using an angled block on a vibratome as described (31). Slices were incubated for 1 h at 32°C in artificial cerebrospinal fluid (ACSF: 126 mM NaCl, 3 mM KCl, 1.25 mM NaH_2PO_4, 26 mM $NaHCO_3$, 2 mM $MgCl_2$, 2 mM $CaCl_2$, and 25 mM d-glucose), followed by perfusion with modified ACSF for 45 min (as above, but with 5 mM KC, 1 mM $MgCl_2$, and 1 mM $CaCl_2$). For drug treatment, 1 or 3 μM of BAER-101 was added during the entire 45 min in modified ACSF. Using 0.5 MΩ tungsten microelectrodes, spontaneously occurring UP states were recorded extracellularly from layer 4 of the primary somatosensory cortex for 10 min, amplified 10,000-fold, sampled at 2.5 kHz, and filtered on-line between 300 Hz and 5 kHz. UP state analysis was done with custom LabVIEW software. Briefly, recordings were offset to 0, rectified and a low-pass filter was set at 0.2 Hz cutoff frequency. The detection threshold was set at 4-fold of the root mean square noise. The beginning of an UP state was defined as events in which the amplitude remained above threshold for at least 200 ms. The end of the UP state was defined as a decrease of the amplitude below the threshold for >600 ms, whereas two events within 600 ms were defined as one single UP state. In the figure, *n* is the number of slices.

Electrode Implanting

Electrode implanting and EEG recording were performed as described (33). Briefly, 6–8 week-old male *Fmr1* KO mice and littermate WT controls were implanted with single-channel wireless transmitters for EEG monitoring [TA11ETA-F10, Data Science International (DSI), St. Paul, MN] under isoflurane anesthesia. Mice were given analgesics (Carprofen) prior to and after the surgery, and surgical sites were disinfected with 2% Chlorhexidine. Dorsoventral coordinates were measured from bregma and two holes were drilled at AP = −2 mm, L = ± 4.0 mm. The two leads of the transmitter were inserted into the burr holes on top of the dura (∼1 mm) and sealed with GLUture (Zoetis Inc., Kalamazoo, MI). The wireless transmitter was placed subcutaneously behind the neck. The assembly was secured with dental cement (Lang Dental, IL). After the cement had dried, the incision was closed using surgical sutures (Coviden, Dublin, Ireland) and sealed with GLUture. Mice were injected with 1 ml saline, placed on a heating pad, and monitored during recovery.

EEG Recording and Analysis

After electrode implantation, mice were housed in individual cages placed on wireless receiver plates (RPC1; DSI). EEG data received from the telemetry system were recorded with DATAQUEST A.R.T software and sampled at 500 Hz, providing readouts for frequencies between 1 and 200 Hz (maximal sampling rate of the wireless transmitter TA11ETA-F10). Video was continuously recorded in parallel (Axis 221, Axis communication) and synchronized with the EEG signal. Daily treatment with 1 mg/kg BAER-101 or vehicle started 9–12 days after the surgery to allow for recovery and lasted for 10 days. Brains were collected 1–2 h after the last drug dose and processed for Golgi-Cox staining (*see below*). EEG data were analyzed with NeuroScore software (DSI) for 9 consecutive days starting at the day of the first dose and ending on the day of the ninth dose. A 5-min period of recording (free of excessive movement and grooming behavior to avoid artifacts) was selected from individual mice within 1–3 h of treatment (∼12–2 pm each day). For EEG power analyses, the raw EEG signal was exported in 10 s epochs and subjected to Fast Fourier Transformation to generate power bands. The data were pooled from 10 s epochs for a total of 5 min duration per day and screened to remove artifacts. Averages from 5 min segments of 9 treatment days are shown. The EEG signal was split into power bands of the following frequencies: delta (δ, 0.5–4 Hz), theta (θ, 4–8 Hz), alpha (α, 8–12 Hz), sigma (Σ, 12–16 Hz), beta (β, 16–24 Hz) and gamma (γ, 24–80 Hz) (34). Power bands were compared between the *Fmr1* KO and WT BAER-101- and vehicle-treated mice. Separate analyses were performed after normalization of individual power bands to total power. A total of 27 mice were implanted with electrodes for this study, 2 died before or during treatment and 2 mice were used for a pilot study trying different drug doses and thus were removed from analysis. Of the remaining 23, 6 mice had to be removed because of EKG signal or highly noisy EEG (1 WT vehicle-treated, 1 WT BAER-101-treated, 2 *Fmr1* KO vehicle-treated and 2 *Fmr1* KO BAER-101-treated mice).

Dendritic Spine Analysis

Dendritic spines from mice that underwent EEG analysis were visualized using the FD Rapid Golgi-Stain Kit from *FD Neurotechnologies, Inc.* (Columbia, MD) as we have done before (35). Briefly, brains were harvested 2–3 h after the last dose of BAER-101 or vehicle, Golgi impregnated, and then cut

into 160 μm thick slices. The slices were stained following the manufacturer's protocol and imaged with a 60x oil objective using a Nikon inverted microscope. Secondary apical dendrites (50–150 μm length, ≥100 μm distant from the soma) of the hippocampal CA1 (bregma −1.8 to −2.2) were analyzed using ImageJ (*NIH*). Eleven to 15 dendrites from five to seven mice per condition were analyzed. Statistical analyses were based on dendrite number. Note that several mice from this cohort could not be used for EEG analyses as stated above but were used for dendritic spine analyses. All mice used for dendritic spine analysis had cortical surface electrodes implanted as described above and were treated with either 1 mg/kg BAER-101 or vehicle for 10 days.

Juvenile Audiogenic Seizure Test

Male *Fmr1* KO and WT littermates were housed with their litter and dam and were treated via oral gavage with vehicle (VEH), 1 mg/kg BAER-101, or 3 mg/kg BAER-101 30 min prior to assessment. The audiogenic seizure test consisted of a 2 min priming tone (120 dB siren), which does not typically induce seizure behavior, followed by 1 min of silence and then a second tone (120 dB siren) lasting an additional 2 min (36). Each mouse was tested alone in a static mouse cage free of bedding. A Mugger Stopper Plus personal alarm was used to generate the tone and was placed on the filter cage lid with the speaker facing down into the cage. The battery was replaced often to ensure the sound intensity was always at maximum. During the second tone, behavioral response was scored as 0, 1, 2, 3, or 4 with 0 indicating no altered behavior, 1 indicating wild-running, 2 indicating clonic seizure (rapid limb flexion and extension), 3 indicating tonic seizure (static limb extension), and 4 indicating the most severe response of death (37). No seizure behavior was observed during the priming tone for this cohort of mice. Seizure severity during the second tone was calculated by using a mouse's most severe response number. Seizure severity was analyzed by the Exact Wilcoxon Rank sum test for non-parametric data. Treatment group (WT+VEH, WT+1BAER, WT+3BAER, KO+VEH, KO+1BAER, KO+3BAER) was used with exact probabilities calculated to determine pairwise group comparisons. These group comparisons were corrected using the false discovery rate (FDR) method.

Adult Behavior Battery

Behavior was assessed during the light portion of the light/dark cycle and food and water were available *ad libitum* except during active behavior testing. Mice began testing on day 11 of treatment. To minimize the impact of stress during behavioral testing, mice were transported across the hallway to the Rodent Behavior Core and dosed with VEH, 1BAER, or 3BAER and allowed 30 min in the testing room to acclimate before behavior assessment. Elevated zero maze was the only exception in which mice were brought into the testing room one at a time just prior to being placed on the maze in order to get an accurate anxiety assessment. Mice were tested in only one paradigm per day, except for locomotor activity and marble burying, which were performed on the same day. Behavior was evaluated in the following order so that tests easily influenced by stress were completed early during the behavior battery: elevated zero maze (EZM), locomotor activity, marble burying, acoustic startle habituation, prepulse inhibition of startle, novel object recognition (NOR), rotarod. We also performed an adhesive removal assay and a pole descend assay as described (38, 39) before the rotarod assay but these experiments did not show genotype or drug effects, have not been shown to be altered in *Fmr1* KO mice before and are thus not reported in the manuscript. Apparatus surfaces were cleaned with Process NPD (Steris) before testing started and between mice. Sample sizes of treatment groups were as follows: WT vehicle: $n = 23$, WT 1BAER: $n = 25$, WT 3BAER: $n = 24$, *Fmr1* KO vehicle: $n = 21$, *Fmr1* KO 1BAER: $n = 20$, *Fmr1* KO 3BAER: $n = 20$. Mice were tested in 3 separate cohorts. Mice that were excluded from the analysis, if any (e.g., because they could not complete the task, were sick or died), are indicated under each assay below.

Elevated Zero Maze

The EZM was used to assess anxiety-like behavior as described with modification of the maze size (40). Briefly, mice were transported from the housing room to the testing room individually and placed on the apparatus. The experimenter exited the room immediately after placing the mouse in one of the closed quadrants of the apparatus. A camera mounted above the maze connected to a computer located outside the room was used to observe and score, in real-time, time in open quadrants and number of open arm entries (transitions during a single 5 min trial) (ODLog, Macropod Software). The test room was dimly lit (30 lux) to encourage exploration of the test environment. One *Fmr1* KO vehicle-treated mouse, 2 *Fmr1* KO 3BAER-treated mice and 2 WT 3BAER-treated mice were excluded from the EZM analysis.

Locomotor Activity

Locomotor activity was measured in infrared photocell activity chambers (41 × 41 cm; PAS Open Field, San Diego Instruments, San Diego, CA) for 1 h. Total Distance was recorded during 5 min intervals for a total of 60 min and analyzed with a 3-way ANOVA with repeated measures. Room lights were at full level (1,200 lux).

Marble Burying

Immediately after spontaneous locomotor activity assessment, mice were moved to an adjacent room and tested in a marble burying task. Through unpublished observations we found that assessing marble burying directly following locomotor activity elicits the most reliable degree of burying in control animals. Briefly, mice were placed in a standard rat cage containing 10 cm (depth) of woodchip bedding. Twenty marbles were evenly distributed on the surface of the bedding using a template in four rows of five. Mice were individually placed in the cage for 10 min and scored for the number of marbles at least 2/3 buried at the end of the testing session.

Novel Object Recognition

A solid black enclosure with dimensions 19.5 cm L × 40 cm W × 35 cm H was used to assess NOR. During the familiarization phase, mice were presented with two identical objects for a total of 5 min. Mice were returned to their cage and left undisturbed

for 30 min. Next, mice were placed back in the enclosure with a novel object and one identical copy of the familiarization phase object. Pilot mice had no inherent preference for any of the objects used in this test (*data not shown*). The amount of time each mouse spent paying attention to the familiar and novel objects during the familiarization and test phases was recorded using OD Log (Macropod Software) for the 5 min duration of each phase. Time spent paying attention was recorded when the mouse was oriented toward the object with snout within 1 cm of the object or when forepaws were up against the object. Mice in these cohorts did not climb on top of the objects used for this test. The discrimination index (DI; novel object time—familiar object time/novel object time + familiar object time) was used to determine the degree of object memory. Dim lighting conditions (20 lux) were used to reduce anxiety and encourage object exploration during both phases. DI during the test phase was analyzed by 2-way ANOVA.

Acoustic Startle Habituation and Prepulse Inhibition (PPI)

Acoustic startle habituation and PPI were assessed in a sound-attenuating test chamber (SR-LAB apparatus; San Diego Instruments, San Diego, CA) as described with modifications (41). Mice were placed in an acrylic cylindrical holder that was mounted on a platform with a piezoelectric force transducer attached to the underside of the platform. For both habituation and PPI, a 5 min acclimation period preceded test trials. For habituation, each mouse received 50 repeated 20 ms 120 dB SPL mixed frequency sound bursts (1.5 ms rise time, analyzed in 5 blocks of 10 trials). Maximum startle amplitude for each trial (Vmax; measured in arbitrary units; a.u.) was analyzed by repeated measures 3-way ANOVA. For PPI, each animal received a 5 × 5 Latin square sequence of trials that were of 5 types: startle stimulus (SS) with no prepulse, 73 dB prepulse + SS, 77 dB prepulse + SS, or 82 dB prepulse + SS. The startle signal was a 20 ms 120 dB SPL mixed frequency sound burst (1.5 ms rise time). Prepulses preceded the startle-eliciting stimulus by 70 ms (onset to onset). The startle recording window was 100 ms. Background noise level was 70 dB. Each set of 25 trials was repeated 4 times for a total of 100 trials. The inter-trial interval averaged 14 s and varied randomly from 8 to 20 s. Vmax at each prepulse level was analyzed by 3-way mixed factor ANOVA with genotype and drug as between factors and PPI trial type as a within factor. Two *Fmr1* KO vehicle-treated mice, 1 WT 1BAER-treated mouse, and 1 WT 3BAER-treated mouse were excluded from PPI and startle analyses.

Rotarod

There were four trials each test day, and 2 days of testing. Mice were tested in bins, so all mice went through trial 1 before starting trial 2. Intertrial interval was a minimum of 10 min. The rod accelerated from 4 to 40 rpm over the first 180 s. The latency to fall from the rod, or a complete full rotation without walking, was recorded as the dependent variable and measured by the computer connected to the laptop. If a mouse did not fall off the rod, the full 300 s score was recorded. The investigator placed the mouse facing the back of the apparatus onto the stationary rod before the test was started.

Extracellular Signal-Regulated Kinase (ERK1/2) Quantification

Following the adult behavior battery, mice continued to be treated for 3–5 additional days prior to sacrifice. Care was taken to minimize stress on the final day of treatment in which dosing occurred 30 min prior to sacrifice. Mice were removed from their cage, which was kept in their permanent housing room and transferred directly to necropsy one at a time. Decapitation occurred within 30 s from removal of the mice from the housing room. Brains were removed and maintained on ice. For ERK1/2 quantifications, the hippocampus was removed from one hemisphere and rapidly frozen onto a stainless-steel plate over dry ice. Once frozen, brain tissue was transferred to a microfuge tube and stored at −80C until assayed. For total protein determination, the hippocampus was homogenized in ice-cold RIPA buffer (500 μl and 100 μl, respectively), with the fresh addition of HALT phosphatase inhibitor cocktail (ThermoScientific) and protease inhibitor cocktail (Sigma) and assayed using the Pierce BCA Protein Assay Kit (ThermoScientific) according to manufacturer's instructions. Samples were diluted to 50 μg/ml for phosphorylated ERK1/2 (pERK1/2) and 2.5 μg/ml for ERK1/2 total prior to analysis. pERK1/2 and ERK1/2 total were analyzed by semiquantitative SimpleStep ELISAs (enzyme-linked immunosorbent assay; ABCAM; phosphoERK1/2 pT202/Y204, ab176640 and ERK1/2 Total, ab176641) according to manufacturer's instructions. Data were verified to fall within the linear range of the standard curve that was run on each plate and mean optical densities of duplicate samples was used for calculations. ERK1/2 total and the ratio of pERK1/2 over ERK1/2 total normalized to WT+VEH were analyzed by 2-way ANOVA with genotype (WT or *Fmr1* KO) and drug (VEH, 1 mg/kg, or 3 mg/kg BAER-101) as factors.

Statistical Analysis

Behavioral data and ERK1/2 phosphorylation were analyzed using mixed linear factorial analysis of variance (ANOVA; Proc Mixed) with the exception of seizure severity score in which the Exact Wilcoxon Rank sum for non-parametric data was used (SAS v9.4, SAS Institute, Cary, NC). Significant main effects and interactions were followed-up with pairwise group comparisons using the False Discovery Rate (FDR) method to control for multiple comparisons (42). Specific details relating to between and within factors and repeated measures were briefly described above with specifics detailed in the Results. Data are shown as least squares (LS) mean ± standard error of the mean (SEM) for model consistency with the exception of seizure severity, EEG power, UP states, and dendritic spine density, in which ordinary means and SEM are shown. UP states and dendritic spine density were analyzed with 2-way ANOVA and EEG data were analyzed with ordinary 2-way ANOVA or mixed-effects analysis (restricted maximum likelihood, REML) because of missing values, followed by Tukey's *post-hoc* tests when significant interactions were observed using GraphPad Prism v8.1 or v9.0. Sample sizes are detailed in the Results section, and/or figure legends. All

behavioral coding, slice analyses, dendritic spine counting, and molecular assays were performed by experimenters blind to genotype and treatment group. A $p < 0.05$ was considered significant and trends are reported at $p < 0.1$. Statistically significant differences in pairwise comparisons are reported in the figure and the figure legends. Details about sample sizes and statistical tests for each experiments are also provided in **Supplementary Table 1**.

RESULTS

Experimental mouse cohorts and the order in which assays were conducted are stated in the method section and differ from how they are discussed below.

BAER-101 Reduces Brain Hyperexcitability in *Fmr1* KO Mice

Individuals with FXS are hyperactive, hypersensitive to auditory and visual stimuli, and have increased gamma frequency band power in resting state dense-array EEGs (10, 11). This cortical hyperexcitability is replicated in the mouse model on brain network and behavioral levels. Because BAER-101, as a $GABA_A$ agonist, is expected to enhance inhibitory signaling, we assessed if the drug reduces hyperexcitable network activity in *Fmr1* KO mice by testing its effect on neocortical UP states, cortical EEG abnormalities, and audiogenic seizures.

BAER-101 Normalizes Prolonged Up States

UP states, a type of persistent activity state of local neocortical circuits, are prolonged in cortical slices of *Fmr1* KO mice, which reflects local circuit hyperexcitability (31, 43). Pre-treatment of *Fmr1* KO cortical slices with 1 μM BAER-101 significantly reduced the duration of UP states in *Fmr1* KO mice compared with vehicle-treated *Fmr1* KO slices [**Figures 1A,B**, 2-way ANOVA, $^*p$(genotype) < 0.0001, $^*p$(drug) $= 0.004$, p(interaction) $= 0.22$; Sidak's multiple comparisons *post-hoc* tests, $^{***}p < 0.001$; $^*p < 0.05$]. UP state amplitude (**Figure 1C**) was not significantly different between WT and *Fmr1* KO slices, but reduced by 1 μM BAER-101 independently of genotype [2-way ANOVA, p(genotype) $= 0.2$, $^*p$(drug) $= 0.015$, p(interaction) $= 0.61$]. UP state frequency (**Figure 1D**) was not significantly different between WT and *Fmr1* KO slices and not affected by 1 μM BAER-101 [2-way ANOVA, p(genotype) $= 0.95$, p(drug) $= 0.71$, p(interaction) $= 0.12$]. Incubation with 3 μM BAER-101 reduced UP state duration even further but also reduced UP state amplitude below WT levels (*data not shown*) and was therefore not further analyzed. These results suggest that selective $GABA_A$ modulation with BAER-101 reduces neocortical circuit hyperactivity in FXS.

Susceptibility to Audiogenic Seizures Is Reduced by Baer-101 in A Dose-Dependent Manner

Juvenile *Fmr1* KO mice are susceptible to audiogenic induced seizures whereas WT mice (C57BL/6 background) of all ages and adult *Fmr1* KO mice are mostly resistant. This increased susceptibility to audiogenic seizures is reminiscent of sensory hypersensitivity in individuals with FXS (44). Analysis of the seizure severity score in response to a 120 dB sound stimulus in 3-week old *Fmr1* KO mice and WT littermates 30 min after one dose of vehicle, 1 mg/kg BAER-101, or 3 mg/kg BAER-101 showed a significant effect of treatment (Kruskal-Wallis statistic: 24.86, $^*p = 0.0001$) (**Figure 1E**). Exact probabilities were computed to determine pairwise comparisons corrected using FDR and revealed significant increases in seizure severity score in the vehicle-treated KO mice compared with all other groups ($^*p < 0.01$ for all comparisons). These data support the previously reported susceptibility to audiogenic seizures in *Fmr1* KO mice and indicate a significant treatment effect in both the low and high dose BAER-101-treated *Fmr1* KO mice (1 and 3 mg/kg BAER-101). The higher dose of BAER-101 on average decreased the seizure score even further compared with 1 mg/kg but there was no statistically significant difference between the two doses (**Figure 1E**). These results suggest that selective $GABA_A$-modulation reduces sensory hyperexcitability in *Fmr1* KO mice.

Low-Dose BAER-101 Reduces Increased Delta Frequency Band Power but Does Not Correct Increased Gamma Frequency Band Power in *Fmr1* KO Mice

Resting gamma EEG power is increased in *Fmr1* KO mice and in humans with FXS (11, 45), suggesting a translationally relevant phenotype. To test the effect of BAER-101 on resting-state brain activity in the absence of FXP, we performed cortical surface EEG recordings from the auditory cortex of *Fmr1* KO mice and WT littermates paired with video recordings for 9 consecutive days during which mice were treated daily with either 1 mg/kg BAER-101 or vehicle. EEG power was analyzed during a 5-min period each day 1–3 h after dosing. The analysis period was chosen based on the video recordings to ensure that mice were sitting still to avoid artifacts caused by grooming or movement. These analyses confirmed that resting gamma power is increased in the auditory cortex of *Fmr1* KO mice [**Figure 2A**, mixed-effects 3-way ANOVA, $^*p$(genotype) $= 0.007$]; but no significant effects of day ($p = 0.30$), treatment ($p = 0.93$), or any interactions (all $p > 0.3$) were observed. Because we did not detect significant effects of day or any significant interactions of day with genotype, drug, or both, we compared the 9-day average with very similar results [**Figure 2B**, 2-way ANOVA, $^*p$(genotype) $= 0.007$; p(treatment) $= 0.98$, p(interaction) $= 0.33$]. To account for variability in signal intensity between mice and to better mimic human EEG analyses we also calculated relative power by normalizing gamma power to total power across all frequencies. While overall trends were the same, no significant effect of genotype on relative gamma power was observed [**Figure 2C**, 2-way ANOVA, p(genotype) $= 0.3$; p(treatment) $= 0.81$; p(interaction) $= 0.45$]. A previous study showed that apart from gamma power, delta power is also increased in the auditory cortex of *Fmr1* KO mice (45). In line with these findings, we observed on average increased delta power in *Fmr1* KO mice compared with WT littermates (**Figures 2D–F**). Analyses per day (**Figure 2D**) and averaged across the 9-day recording period (**Figure 2E**) showed significant

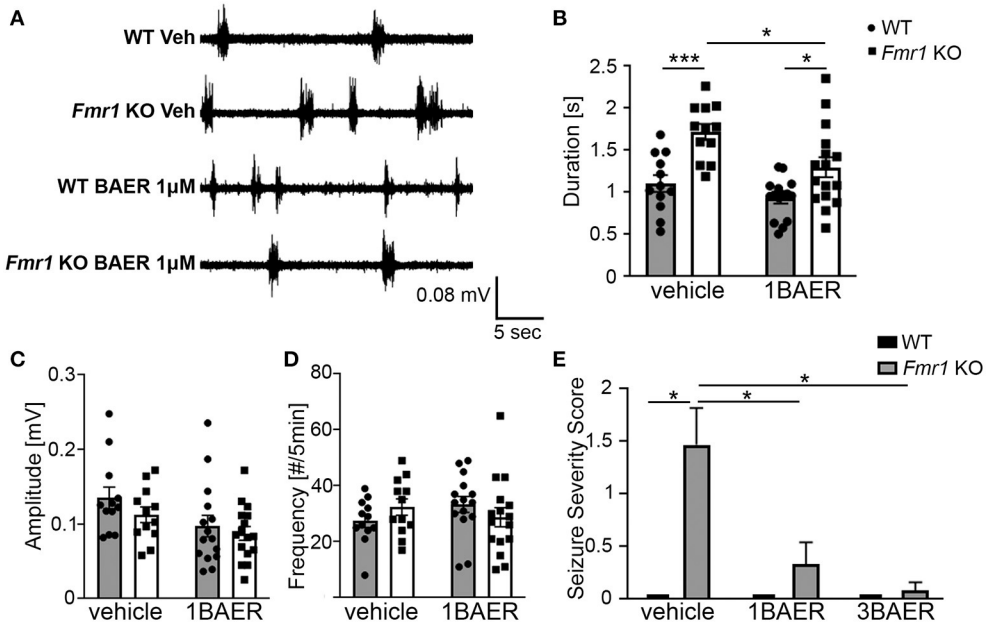

FIGURE 1 | BAER-101 reduces neocortical hyperexcitability and seizures in *Fmr1* KO mice. **(A–D)** Increased duration of spontaneous UP states in neocortical slices from *Fmr1* KO mice is reduced to WT levels by bath application of 1 μM BAER-101. **(A)** Representative extracellular multi-unit recordings from layer IV of acute neocortical slices prepared from WT or *Fmr1* KO mice and preincubated for 1–1.5 h in either BAER-101 (1 μM) or vehicle (0.03% DMSO). **(B)** Increased duration of UP states in *Fmr1* KO slices is reduced by BAER-101 [2-way ANOVA with Sidak's multiple comparison, p(genotype) < 0.0001, p(drug) = 0.004, p(interaction) = 0.22]; ***$p < 0.001$; *$p < 0.05$. **(C)** Amplitude of UP states was reduced by BEAR-101 but not affected by genotype and no interaction was detected [2-way ANOVA, p(genotype) = 0.2, *p(drug) = 0.015, p(interaction) = 0.61]. **(D)** UP state frequency was not affected by genotype or treatment [2-way ANOVA, p(genotype) = 0.95, p(drug) = 0.71, p(interaction) = 0.12]. Sample sizes for UP states were as follows (slices): WT vehicle: $n = 12$, WT 1BAER: $n = 15$, *Fmr1* KO vehicle: $n = 12$, *Fmr1* KO 1BAER: $n = 16$. **(E)** Audiogenic seizures are significantly reduced by administration of 1 mg/kg and 3 mg/kg BAER-101 (1BAER and 3BAER, respectively), 30 min before testing (Kruskal-Wallis test, $p = 0.0001$; Wilcoxon rank pair-wise comparisons with FDR correction: *$p < 0.01$). Quantification of seizure scores suggests a dose-dependent effect with stronger reduction with 3 mg/kg BAER-101. WT vehicle: $n = 12$, WT 1BAER: $n = 10$, WT 3BAER: $n = 11$, *Fmr1* KO vehicle: $n = 17$, *Fmr1* KO 1BAER: $n = 15$, *Fmr1* KO 3BAER: $n = 13$.

effects of treatment, reducing delta power independently of genotype, but no other effects [**2D**, 3-way ANOVA, all $p >$ 0.3, except for *p(treatment) = 0.047; **2E**, 2-way ANOVA, p(genotype) = 0.30, p(interaction) = 0.50, *p(treatment) = 0.045]. This suggests a selective effect of 1 mg/kg BAER-101 on delta but not gamma EEG power. Of note, there was a trend toward a significant interaction between genotype and treatment for relative delta power [**Figure 2F**, 2-way ANOVA p(interaction) = 0.10] and a significant reduction of relative delta EEG power in BAER-101 treated *Fmr1* KO mice compared with vehicle-treated *Fmr1* KO mice but not for WT mice, suggesting that the drug differentially affects relative delta power depending on genotype (**Figure 2F**, Tukey *post-hoc* test, *p = 0.04). As reported previously (45), none of the other frequency bands' power (relative or absolute) was significantly different in the auditory cortex of *Fmr1* KO mice compared with WT littermates (theta, alpha, sigma) with the exception of absolute (but not relative) beta power, which was overall increased in *Fmr1* KO mice [*p(genotype) = 0.03] with no effect of treatment [p(treatment) = 0.38] (**Supplementary Figure 1**). Overall, these studies support previous findings of altered cortical activity in *Fmr1* KO mice and suggest that 1 mg/kg BAER-101 frequency band-specifically reduces increased resting brain EEG power in the absence of FXP.

Short-Term Treatment of Adult Mice With Low-Dose BAER-101 Does Not Correct Increased Dendritic Spine Density in *Fmr1* KO Mice

Dendritic spine density is increased and dendritic spine morphology altered in humans with FXS and in *Fmr1* KO mice, which may contribute to the observed brain circuit defects discussed above (46). We tested whether daily dosing with 1 mg/kg BAER-101 for 10 days normalizes dendritic spine density in the hippocampal CA1 region. We confirmed increased dendritic spine density on secondary apical dendrites of *Fmr1* KO mice compared with WT littermates, but did not detect an effect of treatment [**Figure 3**, 2-way ANOVA, *p(genotype) = 0.001; p(treatment) = 0.90; p(interaction) = 0.72]. We speculate that longer dosing, and thus longer-term modulation of $GABA_A$, is necessary to correct dendritic spine density in *Fmr1* KO mice.

BAER-101 Alters Select FXS-Specific Behavioral Phenotypes

To evaluate the effects of BAER-101 treatment on behavioral phenotypes in *Fmr1* KO mice and WT littermates, we

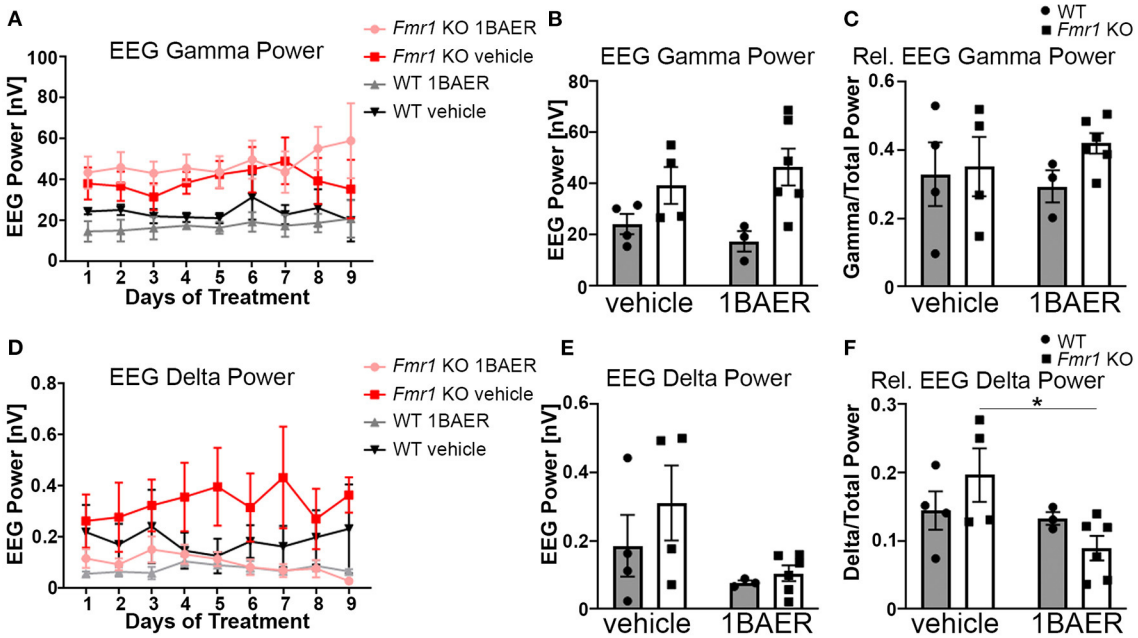

FIGURE 2 | Low-dose BAER-101 reduces increased delta EEG power but not gamma EEG power in *Fmr1* KO mice. **(A–C)** Gamma EEG power is increased in *Fmr1* KO mice but not affected by daily treatment with 1 mg/kg BAER-101 (1BAER) over 9 days. The effect of drug or genotype on absolute gamma EEG power does not change during the 9 day treatment period [**A**, separated by day, mixed-effects analysis, p(genotype) = 0.007, p(day) = 0.30, p(day x treatment) = 0.72; p(day x genotype) = 0.99, p(day x treatment x genotype) = 0.80; no other significant effects; **(B)**, 9-day average, 2-way ANOVA, p(treatment) = 0.98, p(genotype) = 0.007, p(interaction) = 0.33]. Relative gamma power (normalized to the EEG power across all frequency bands) shows on average non-significantly increased power in *Fmr1* KO mice compared with WT [**C**, 9-day average, 2-way ANOVA, p(treatment) = 0.81, p(genotype) = 0.29, p(interaction) = 0.45]. **(D–F)** Delta EEG power is reduced by daily treatment with 1 mg/kg BAER-101 over 9 days. Similarly as for gamma EEG power, the effect of drug or genotype on absolute delta power does not change over the 9-day period [**D**, separated by day, mixed-effects analysis, p(treatment) = 0.047, p(day) = 0.58, p(day x treatment) > 0.99; p(day x genotype) = 0.40, p(day x treatment x genotype) = 0.31; no other significant effects; **(E)** 9-day average, 2-way ANOVA, p(treatment) = 0.045; p(genotype) = 0.30, p(interaction) = 0.50]. Relative delta power in *Fmr1* KO is significantly reduced by 1 mg/kg BAER-101 whereas no effect on WT was observed [**F**, 2-way ANOVA with Sidak's *post-hoc* test, p(treatment) = 0.046; p(genotype) = 0.87, p(interaction) = 0.10; $^*p = 0.04$]. WT vehicle: $n = 4$; *Fmr1* KO vehicle: $n = 4$; WT 1BAER: $n = 3$; *Fmr1* KO 1BAER: $n = 6$. EEG power was analyzed during a 5 min period within 1–3 h after drug dosing (~12–2 pm each day). Analysis of other waveforms is shown in **Supplementary Figure 1**.

treated adult mice daily with 1 mg/kg or 3 mg/kg BAER-101 or vehicle. Daily treatment begun 10 days before the start of behavioral assays and continued throughout the behavioral assessments. The order of behaviors is stated in the methods and differed from how they are presented here. The completion of the behavioral battery lasted between 2 and 3 weeks for all cohorts, during which time daily treatment continued.

BAER-101 Increases Locomotor Activity in the Open Field

To assess if $GABA_A$ modulation by BAER-101 affects locomotor activity and coordination, we performed open field analyses and rotarod assays (**Figure 4**). Overall, we detected no or only genotype-unspecific effects of BAER-101 on these measures.

High-Dose BAER-101 Increases Locomotor Activity

Activity analysis in an open field, an overall indication of an animal's activity level, is sensitive to sedative drugs (including GABA modulators) or those inducing stereotypy or catatonia and is especially useful in better interpreting other tasks that depend on the overall activity of the animal. We therefore tested all mice in the open field for 60 min, separated into twelve 5-min intervals for analysis. In summary, all mice were more active in the beginning of the testing session, *Fmr1* KO mice were overall more active [as we and others reported before (47)], and BAER-101 treatment increased activity further (**Figure 4A**, 3-way repeated measures ANOVA, main effects of interval, genotype, and treatment: $^*p < 0.0001$). Apart from a significant treatment x interval interaction ($^*p < 0.0001$) there were no other significant interactions, indicating no genotype-specific treatment effect. Although vehicle-treated *Fmr1* KO mice on average traveled further than vehicle-treated WT littermates, there was only a significant difference between *Fmr1* KO mice treated with 3 mg/kg BAER-101 and vehicle-treated WT littermates in pairwise comparisons [**Figure 4B**, FDR-corrected pairwise comparisons on data collapsed over time, p(wt/veh-ko/veh) = 0.12; $^*p = 0.002$]. These data suggest that high-dose BAER-101 causes elevations in activity levels when *Fmr1* KO mice are placed in a novel environment.

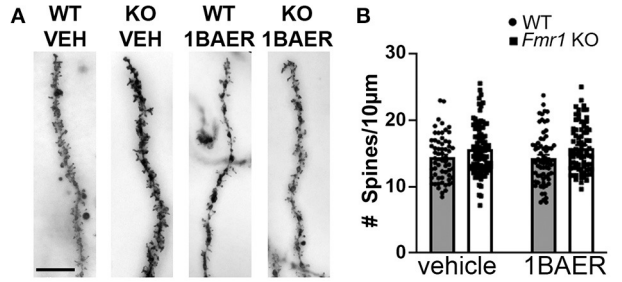

FIGURE 3 | Increased dendritic spine density in *Fmr1* KO hippocampus is not affected by short-term treatment with low-dose BAER-101. Dendritic spine density on apical CA1 hippocampal dendrites from *Fmr1* KO and WT mice after 10 days of daily treatment with vehicle or 1 mg/kg BAER-101 (1BAER) was visualized by Golgi staining and quantified by manual counting using ImageJ. Representative images are shown in **(A)** quantifications in **(B)**. Dendritic spine density is increased in *Fmr1* KO mice but not changed by BAER-101 treatment [2-way ANOVA, p(treatment) = 0.87; p(genotype) = 0.0013, p(interaction) = 0.72]. Scale bar is 10 µm. WT vehicle: $n = 61$ dendrites from 5 mice (12–13 dendrites each); *Fmr1* KO vehicle: $n = 94$ dendrites from 7 mice (13–16 dendrites each); WT 1BAER: $n = 67$ dendrites from 5 mice (13–14 dendrites each); *Fmr1* KO 1BAER: $n = 78$ dendrites from 6 mice (11–15 dendrites each).

BAER-101 Does Not Affect Motor Coordination in Rotarod Experiments

To assess the effect of *Fmr1* gene deletion and BAER-101 on motor coordination, we used a rotarod test. In this test mice have to walk and balance on a horizontal rod that rotates around its own axis. The assay is performed twice, on two consecutive days, and the time to fall is used as a measure for motor coordination (48). As expected, all mice improved from day 1 (129.34 s +/– 4.35 s) today 2 (171.78 s +/– 4.35 s) [3-way repeated measures ANOVA; p(day) < 0.0001]. We observed that, overall, *Fmr1* KO mice fell off the rod earlier than WT mice [$^*p$(genotype) = 0.044]; however there was no main effect of drug and no interaction effects indicating that BAER-101 did not affect motor coordination (**Figure 4C**, average data across both days are shown).

BAER-101 Alters Anxiety-Related Behavior in Mice

We used the EZM assay to assess anxiety behavior in vehicle- and BAER-101-treated *Fmr1* KO and WT mice during a 5-min test. Time in the open arm and number of transitions between the open and closed arm (= open arm entries) were measured (**Figures 5A,B**). We detected significant increases for time in open and open arm entries in the *Fmr1* KO mice compared with WT mice [2-way ANOVAs, $^*p$(genotype) < 0.0001 and 0.0007, respectively]. Neither low- nor high-dose BAER-101 affected time in open [p(treatment) = 0.13, p(interaction) = 0.32] but BAER-101 treatment increased the open arm entries [$^*p$(treatment) = 0.047, p(interaction) = 0.056]. This effect was driven by the high-dose (3 mg/kg) BAER-101 treatment group: *Fmr1* KO mice made significantly more transitions after 3 mg/kg BAER-101 compared with vehicle-treated *Fmr1* KO mice and vehicle-treated WT littermates (FDR-corrected pairwise comparisons, $^*p = 0.019$ and 0.002, respectively). We speculate that increased activity in *Fmr1* KO mice after treatment with high-dose BAER-101, as seen in the open field test, influenced the *Fmr1* KO phenotype in EZM behavior.

BAER-101 Does Not Reverse Increased Repetitive Behavior in *Fmr1* KO Mice

Marble burying is used to gauge repetitive behavior, and *Fmr1* KO mice exhibit increased burying compared with WT mice indicating enhanced repetitive behavior (49). We therefore tested the effect of BAER-101 treatment on this phenotype. After 10 min, *Fmr1* KO mice in general buried more marbles than their WT littermates, as expected, and 1 mg/kg but not 3 mg/kg BAER-101 increased the number of buried marbles in *Fmr1* KO mice further [**Figure 5C**; 2-way ANOVA, $^*p$(genotype) = 0.008; $^*p$(interaction) = 0.031; FDR-corrected pairwise comparison $^*p$(ko/veh-ko/1BAER) = 0.008, $^*p$(wt/1BAER-ko/1BAER) = 0.008, p(ko/veh-ko/3BAER) = 0.42].

BAER-101 May Improve Impaired Memory in *Fmr1* KO Mice in the Novel Object Recognition Assay

Impaired novel object recognition in *Fmr1* KO mice was observed by others (50) and may reflect cognitive deficits associated with FXS. To assess the effect of BAER-101 on this phenotype we determined the time mice spent with a familiar and a novel object in a short-term object recognition test (51). A discrimination index (DI) was used to quantify novel object memory. All groups spent more time with the novel object as indicated by a DI greater than zero, but vehicle-treated *Fmr1* KO mice performed worse than WT littermates [**Figure 5D**, 2-way ANOVA with FDR-corrected pairwise comparisons, $^*p$(gene) = 0.028; p(interaction) = 0.073; $^*p = 0.004$]. By contrast, *Fmr1* KO mice treated with either 1 mg/kg or 3 mg/kg BAER-101 were not significantly different from vehicle-treated WT littermates ($p = 0.87$ and $p = 0.11$, respectively), and 1 mg/kg BAER-101-treated *Fmr1* KO mice showed on average increased (i.e., improved) DI compared with vehicle-treated *Fmr1* KO mice (62.1+/−1.8 vs. 57.7+/−1.7). These results confirm previous studies showing that *Fmr1* KO mice are impaired in short-term object recognition memory and suggest that low-dose BAER-101 may improve this phenotype.

BAER-101 Does Not Alter Sensory Gating or ERK1/2 Activation

Prepulse inhibition (PPI) is a test of startle reactivity and sensorimotor gating and is impaired in young males with FXS, but enhanced in adult male mice (52). Although the reasons for these discrepancies between species are unknown, these previous studies suggest that both mice and people lacking FXP exhibit aberrant sensorimotor gating (52, 53). We therefore assessed how BAER-101 affects PPI in *Fmr1* KO mice. To acclimate the mice to the chamber and sound used for PPI, acoustic startle habituation was performed. All mice habituated to the sound as expected [2-way ANOVA, $^*p$(burst block) < 0.0001], and there was no effect of either 1 or 3 mg/kg BAER-101 treatment ($p = 0.44$) or genotype ($p = 0.28$), and no significant interaction ($p = 0.41$). After the acclimatization phase, PPI was assessed for each mouse

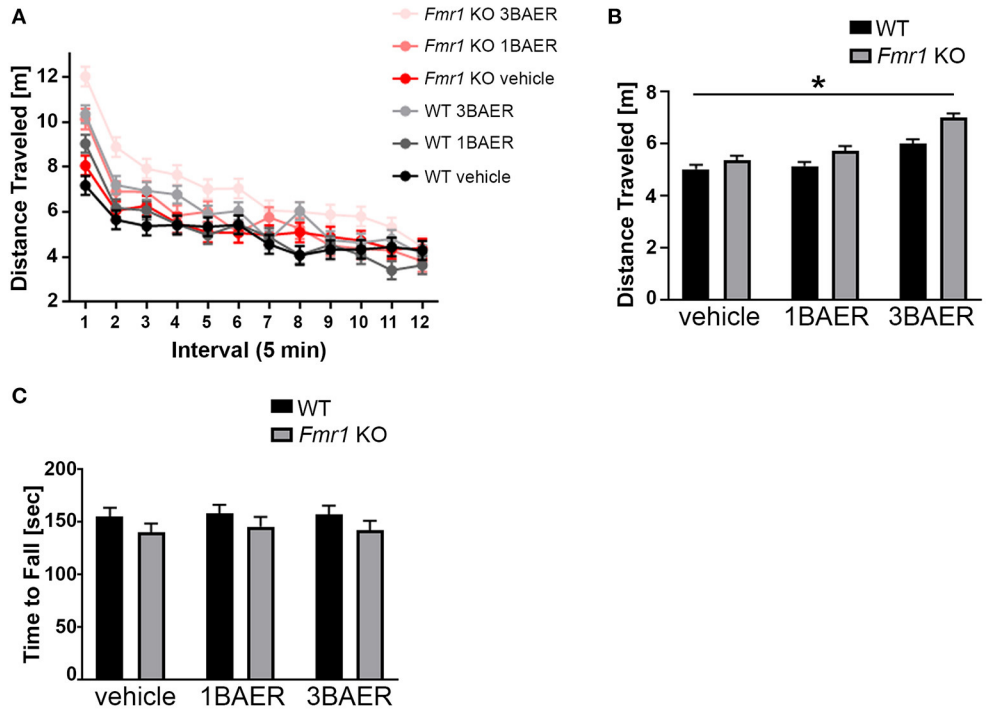

FIGURE 4 | BAER-101 increases motor activity regardless of genotype but does not affect motor coordination. **(A,B)** Distance traveled in an open field is overall increased in *Fmr1* KO mice compared with WT littermates and further increased by BAER-101 treatment [**A**, 3-way ANOVA, p(genotype) < 0.0001, p(treatment) < 0.0001, p(interval) < 0.0001, p(treatment x interval) < 0.0001, p(treatment x genotype) = 0.16, p(treatment x interval x genotype) = 0.79]. FDR-corrected pairwise comparisons of data collapsed over time show significant differences for WT vehicle compared with *Fmr1* KO 3 mg/kg BAER-101 (**B**, $^*p = 0.0015$). **(C)** *Fmr1* KO mice fell off the rotarod faster than their WT littermates but both genotypes improved over time. No effect of low- or high-dose BAER-101 treatment was detected [3-way ANOVA, p(genotype) = 0.044, p(treatment) = 0.87, p(day) < 0.0001, p(genotype x treatment) = 0.98, p(genotype x day) = 0.70, p(treatment x day) = 0.33, p(genotype x treatment x day) = 0.71]. Figure in **(C)** shows data collapsed over 2 days. WT vehicle: $n = 23$, WT 1BAER: $n = 25$, WT 3BAER: $n = 24$, *Fmr1* KO vehicle: $n = 21$, *Fmr1* KO 1BAER: $n = 20$, *Fmr1* KO 3BAER: $n = 20$.

at each of the prepulse trial types (PPI0, PPI73, PPI77, PPI82, numbers are indicating dB for each trial). These experiments did not replicate the previously described increase in PPI in *Fmr1* KO mice and no effect of treatment was observed [3-way mixed effect ANOVA, p(genotype) = 0.89, p(drug) = 0.91, $^*p$(type) < 0.0001, p(genotype x drug) = 0.32, p(genotype x type) = 0.13, p(drug x type) = 0.97, p(genotype x drug x type) = 0.09]. The results of these experiments should be interpreted with caution as the PPI response of the mice was very weak and all prepulses essentially elicited the same reduction in startle response.

To evaluate if BAER-101 corrects molecular defects, such as altered cellular signaling, in the FXS mouse model, we used phospho-ERK1/2- and ERK1/2-specific ELISAs to quantify ERK1/2 phosphorylation in hippocampal lysates from the mice after they underwent the behavioral testing. In a previous study we showed that ERK phosphorylation is increased in the hippocampus of *Fmr1* KO mice (47); however, here, we only detected a trend of increased pERK/ERK in *Fmr1* KO hippocampus and no significant interaction or drug effects [n = 8–10 per group, 2-way ANOVA, p(genotype) = 0.09, p(treatment) = 0.23, p(interaction) = 0.73]. Neither low- nor high-dose BAER-101 significantly changed ERK1/2 phosphorylation in the mice (**Supplementary Figure 2**). We assume that the relative absence of this molecular phenotype could have been caused by the four or more weeks of treatment, behavior testing, and daily handling. Indeed, while many studies demonstrated that neurotransmitter receptor-dependent cellular signaling is altered in FXS, there are partially contradictory findings regarding the steady-state activity of certain pathways, which are mostly attributed to differences in mouse and tissue handling (54). Stress can have significant effects on gene expression (55) most likely altering the molecular pathways that are changed in *Fmr1* KO mice (e.g., ERK1/2, PI3K/mTOR, and GSK3α/β signaling), which could confound molecular analyses. We therefore abstained from analyzing other FXS-associated molecular defects in these mice.

DISCUSSION

Novel disease mechanism-targeted treatments for FXS are urgently needed. A hallmark of FXS is an overall hyperexcitable brain network, which may be partially caused by impaired inhibition through GABAergic signaling. Here, we tested a novel therapeutic strategy in a mouse model of FXS targeting a subset of $GABA_A$ receptors. GABAergic signaling has long

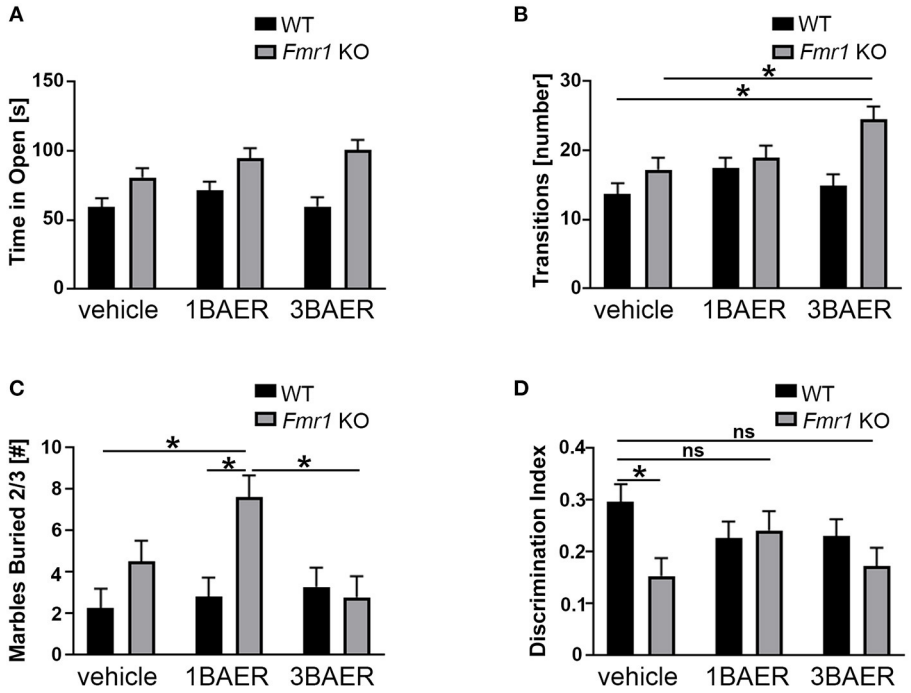

FIGURE 5 | BAER-101 may worsen anxiety-related and repetitive behaviors but improve memory in *Fmr1* KO mice. **(A,B)** *Fmr1* KO mice spent more time in the open **(A)** and made more transitions **(B)** than WT littermates in the elevated zero maze; BAER-101 does not affect time in the open but 3 mg/kg BAER-101 increases the number of transitions between open and closed compartments [**A**, 2-way ANOVA, p(genotype) < 0.0001, p(treatment) $= 0.13$, p(interaction) $= 0.32$; **B**, 2-way ANOVA with FDR-corrected pairwise comparisons, p(genotype) $= 0.0007$, p(treatment) $= 0.047$, p(interaction) $= 0.056$, *p(wt/veh-ko/3BAER) $= 0.0015$, *p(ko/veh-ko/3BAER) $= 0.019$]. **(A,B)** WT vehicle: $n = 23$, WT 1BAER: $n = 25$, WT 3BAER: $n = 23$, *Fmr1* KO vehicle: $n = 19$, *Fmr1* KO 1BAER: $n = 20$, *Fmr1* KO 3BAER: $n = 20$. **(C)** Increased marble burying behavior in *Fmr1* KO mice is enhanced by 1 mg/kg BAER-101 [2-way ANOVA with FDR-corrected pairwise comparisons, p(genotype) $= 0.008$, p(treatment) $= 0.066$, p(interaction) $= 0.03$, *p(wt/veh-ko/1BAER) $= 0.005$, *p(wt/1BAER-ko/1BAER) $= 0.008$, *p(ko/1BAER-ko/3BAER) $= 0.008$). Number of marbles buried by two thirds or more after 10 min is shown. **(D)** 1 mg/kg BAER-101 may improve impaired novel object recognition memory in *Fmr1* KO mice [2-way ANOVA with FDR-corrected pairwise comparisons, p(genotype) $= 0.028$, p(treatment) $= 0.62$, p(interaction) $= 0.073$, *p(wt/veh-ko/veh) $= 0.040$, all other pairwise comparison not significant]. Shown is the discrimination index DI [(time with the novel object−-time with familiar object)/(time with the novel object + time with the familiar object)]. **(C,D)** WT vehicle: $n = 23$, WT 1BAER: $n = 25$, WT 3BAER: $n = 24$, *Fmr1* KO vehicle: $n = 21$, *Fmr1* KO 1BAER: $n = 20$, *Fmr1* KO 3BAER: $n = 20$. *indicates a significant difference, *ns* indicates not significant.

been suggested as treatment target in FXS, but so far, preclinical and clinical studies mostly targeted broad spectrum $GABA_A$ and $GABA_B$ receptors, with mixed successes. Our strategy is novel since we used an investigational drug, BAER-101 that selectively targets only two (out of 19 possible) $GABA_A$ receptor subunits, α2 and α3. Our studies suggest that BAER-101 can reverse neuronal circuit hyperactivity and improve memory in FXS but is ineffective in correcting hyperactivity and repetitive behavior in an FXS mouse model.

Several of our results indicate that BAER-101 at least partially corrects altered inhibitory neuronal transmission in *Fmr1* KO mice. First, in an *in vitro* approach, we showed that bath application of BAER-101 normalizes prolonged duration of UP states, suggesting that neocortical hyperactivity is normalized with the treatment. Second, we showed that BAER-101 significantly reduces the susceptibility to audiogenic seizures in a dose-dependent manner. This suggests that hypersensitivity to sensory stimuli, which is also seen in humans with FXS and most likely reflects a hyperactive and hyperexcitable neuronal network, is corrected by BAER-101. Third, we showed that BAER-101 normalizes enhanced delta EEG power. Enhanced EEG power of select frequency bands can be observed in mice and humans with no or very low levels of FXP, is believed to reflect neocortical hyperexcitability and may serve as a translational biomarker (10, 11, 45). Delta EEG power is associated with cognitive processing and believed to suppress networks not involved in a certain task (56). Notably, the correction of enhanced delta power was associated with improvement in novel object recognition memory, suggesting that correction of EEG power alterations in FXS is a valuable treatment goal.

In contrast to delta EEG power, increased gamma EEG was not rescued by BAER-101. Apart from being an agonist for the α2 and α3 $GABA_A$ receptor subunits, BAER-101 also has neutral antagonistic action toward α1 (AstraZeneca, *personal communication*), which could influence its effect on EEG power bands. Moreover, we evaluated EEG power only during a 9-day treatment period. We speculate that longer treatment is necessary for a more comprehensive rescue of EEG power deficits, as well as for improvement of the dendritic spine phenotype which was likewise not rescued by this treatment paradigm. Lastly, we limited the EEG and dendrite studies to the low-dose BAER-101 condition. This decision was made following analysis of the

behavioral data that indicated potentially enhanced efficacy of low- vs. high-dose drug in the KO mouse; however, we cannot exclude that the higher dose BAER-101 (3 mg/kg) would have been more effective in correcting alterations in gamma EEG power and dendritic spine density in *Fmr1* KO mice. This is particularly relevant, as gamma EEG oscillations are important for sensory processing (57), and audiogenic seizures, a form of hyperresponsivity to sensory stimuli, were most effectively reduced with 3 mg/kg BAER-101. It is thus conceivable that a higher dose of BAER-101 would have been necessary to rescue increased gamma EEG power. A limitation of the EEG analyses in the current study is the low number of animals tested. Future studies with larger sample numbers are needed to draw definitive conclusions.

A benefit of the selective action of BAER-101 on α2 and α3 $GABA_A$ receptor subunits is its lack of sedative effects, which usually limits the clinical usability of broad $GABA_A$ agonists such as benzodiazepines in persons with developmental disability. In fact, we observed *increased* activity in BAER-101-treated mice. Mice also spent more time in the open in the EMZ, suggesting reduced anxiety, which is in line with previous reports that α2 and α3 subunits mediate the anxiolytic effects of unselective GABA receptor agonists (58, 59), but is not consistent with normalization of *Fmr1* KO mouse behavior in this assay thus potentially limiting the face validity of this behavior test. A limitation of our study is that we cannot exclude that the drug-induced increase in activity altered other behavioral phenotypes tested. Current and future clinical trials will have to carefully monitor the effects of BAER-101 on hyperactivity-related symptoms in individuals with FXS.

The increased activity in BAER-101-treated mice may have contributed to the appearance of potentially worsening of the repetitive behavior in the marble burying assay in mice treated with low-dose BAER-101 and could have masked a potential beneficial effect on these repetitive/perseverative behaviors often associated with autism. A recent study supports this notion by showing that $GABA_A$, but not $GABA_B$ receptor agonism reduces marble burying behavior in WT mice (60), corroborating a potential beneficial effect of BAER-101 on perseverative behaviors. Interestingly, no increase in marble burying was observed with the higher dose of 3 mg/kg BAER-101. Instead, the 3 mg/kg BAER-101 appeared to normalize the phenotype (although no statistical significance was reached). This suggests that higher doses are needed to rescue repetitive behavior. We speculate that the opposing effects of low- and high-dose BAER-101 on marble burying may be due to the α1 antagonistic effect of BAER-101 that could have different influences on this phenotype depending on drug dose. In the future, it will be important to assess other autistic-like phenotypes in BAER-101 treated *Fmr1* KO mice to further evaluate its potential to ameliorate autism disorders in FXS. Additionally, different doses of BAER-101 could be evaluated to minimize hyperactivity-inducing actions of the drug.

It is worth noting that the *Fmr1* KO phenotypic representation is subtle and dependent on genetic background and environment, leading to contradictory phenotypes between different laboratories. In this study, we were not able to consistently replicate previously reported changes in PPI or ERK1/2 phosphorylation in *Fmr1* KO mice, and the effect of BAER-101 treatment on these phenotypes could thus not be determined reliably. Moreover, some of the effects we observed were subtle and our assays may have not been sufficiently powered or sensitive enough to detect significant changes. Future studies with larger sample size may be necessary to further analyze the effects of BAER-101 on these phenotypes. Nevertheless, the promising results in brain hyperactivity (UP states, audiogenic seizures, certain EEG frequency bands) and novel object recognition justify further evaluation in preclinical studies and clinical trials.

Based on our results in the mouse model, we predict that low-dose BAER-101 may have a beneficial effect on circuit excitability, sensory hypersensitivity, and cognitive function in FXS. The $GABA_B$ receptor subunit-selective agonist arbaclofen did not meet social end point criteria in a large clinical trial (24). It will thus be interesting to test in follow-up studies how BAER-101 affects phenotypes of sociability [e.g., social novelty or social preference (61)] in *Fmr1* KO mice. It will be important in first-in-human studies to confirm whether low-dose BAER-101 shows a preferential positive clinical effect consistent with this preclinical report.

There are many different $GABA_A$ receptor subunits expressed in the brain that can exist in a variety of different receptor combinations. The proportion of α receptor subunits within $GABA_A$ receptors depends on the brain region and can affect receptor function (62). Our studies suggest that $GABA_A$ agonists selective for specific α subunits improve certain phenotypes in *Fmr1* KO mice. The mRNA levels of several $GABA_A$ subunits are downregulated in *Fmr1* KO mouse cortex (α1, α3, α4, β1, β2, γ1, and γ2) (20). While we did not analyze $GABA_A$ subunit expression in our cohort of mice, this previous observation provides justification for assessing agonists specific to one or more of these $GABA_A$ subunits as disease-targeted treatment in FXS. In selecting which subunit(s) to target it is important to consider previous studies suggesting that some of the $GABA_A$ subunits shown to be altered in the *Fmr1* KO mouse model have functions that make them less attractive drug targets in FXS. For example, both α4 and β1 subunits play a role in alcohol intake and binge drinking (63, 64), increasing the risk of addiction. Moreover, the α1 subunit mediates sedative effects but not anxiolytic effects of benzodiazepines (65). By contrast, the α2/3-selective agonist BAER-101 was shown to be anxiolytic but not sedative making it a preferred candidate as novel therapeutic strategy. Nevertheless, future studies are necessary to evaluate which GABA receptor subunits (or what combinations thereof) are the most beneficial to modulate for the treatment of FXS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committees of Cincinnati Children's Hospital Medical Center and University of Texas Southwestern.

AUTHOR CONTRIBUTIONS

TS conceived, designed, analyzed, coordinated the behavior studies, wrote a first draft of the results, and methods section of the manuscript. AA, CR, and MD performed the ERK1/2 experiments. AA carried out the behavior studies along with MD, RB, and LG. CG, DT, and AW conceived and designed the EEG and dendritic spine studies. DT performed the EEG surgeries and recordings. AW and CG collected tissue samples thereafter. DT, AS, RM, and CG analyzed the EEG experiments. EP performed the Golgi staining, imaging, and dendritic spine analysis. MT, JG, and KH performed and analyzed the slice electrophysiology experiments. MW analyzed some of the behavior data. CG wrote the full draft of the manuscript. CE participated in the conceptualization of this study specifically, was responsible for bringing the BAER-101 molecule into the FXS field of study and edited the manuscript. All authors read, edited, and/or approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.678090/full#supplementary-material

REFERENCES

1. Salcedo-Arellano MJ, Dufour B, McLennan Y, Martinez-Cerdeno V, Hagerman R. Fragile X syndrome and associated disorders: clinical aspects and pathology. *Neurobiol Dis.* (2020) 136:104740. doi: 10.1016/j.nbd.2020.104740
2. Erickson CA, Davenport MH, Schaefer TL, Wink LK, Pedapati EV, Sweeney JA, et al. Fragile X targeted pharmacotherapy: lessons learned and future directions. *J Neurodev Disord.* (2017) 9:7. doi: 10.1186/s11689-017-9186-9
3. Berry-Kravis EM, Lindemann L, Jønch AE, Apostol G, Bear MF, Carpenter RL, et al. Drug development for neurodevelopmental disorders: lessons learned from fragile X syndrome. *Nat Rev Drug Discov.* (2018) 17:280–99. doi: 10.1038/nrd.2017.221
4. Erickson CA, Kaufmann WE, Budimirovic DB, Lachiewicz A, Haas-Givler B, Miller RM, et al. Best practices in fragile X syndrome treatment development. *Brain Sci.* (2018) 8:224. doi: 10.3390/brainsci8120224
5. Gross C, Hoffmann A, Bassell GJ, Berry-Kravis EM. Therapeutic strategies in fragile X syndrome: from bench to bedside and back. *Neurotherapeutics.* (2015) 12:584–608. doi: 10.1007/s13311-015-0355-9
6. Dahlhaus R. Of men and mice: modeling the fragile X syndrome. *Front Mol Neurosci.* (2018) 11:41. doi: 10.3389/fnmol.2018.00041
7. Contractor A, Klyachko VA, Portera-Cailliau C. Altered neuronal and circuit excitability in fragile X syndrome. *Neuron.* (2015) 87:699–715. doi: 10.1016/j.neuron.2015.06.017
8. Leung HT, Ring H. Epilepsy in four genetically determined syndromes of intellectual disability. *J Intellect Disabil Res.* (2013) 57:3–20. doi: 10.1111/j.1365-2788.2011.01505.x
9. Rais M, Binder DK, Razak KA, Ethell IM. Sensory processing phenotypes in fragile X syndrome. *ASN Neuro.* (2018) 10:1759091418801092. doi: 10.1177/1759091418801092
10. Ethridge LE, White SP, Mosconi MW, Wang J, Pedapati EV, Erickson CA, et al. Neural synchronization deficits linked to cortical hyper-excitability and auditory hypersensitivity in fragile X syndrome. *Mol Autism.* (2017) 8:22. doi: 10.1186/s13229-017-0150-z
11. Wang J, Ethridge LE, Mosconi MW, White SP, Binder DK, Pedapati EV, et al. A resting EEG study of neocortical hyperexcitability and altered functional connectivity in fragile X syndrome. *J Neurodev Disord.* (2017) 9:11. doi: 10.1186/s11689-017-9191-z
12. Paluszkiewicz SM, Martin BS, Huntsman MM. Fragile X syndrome: the GABAergic system and circuit dysfunction. *Dev Neurosci.* (2011) 33:349–64. doi: 10.1159/000329420
13. Cea-Del Rio CA, Huntsman MM. The contribution of inhibitory interneurons to circuit dysfunction in fragile X syndrome. *Front Cell Neurosci.* (2014) 8:245. doi: 10.3389/fncel.2014.00245
14. Martin BS, Corbin JG, Huntsman MM. Deficient tonic GABAergic conductance and synaptic balance in the fragile X syndrome amygdala. *J Neurophysiol.* (2014) 112:890–902. doi: 10.1152/jn.00597.2013
15. Goetz T, Arslan A, Wisden W, Wulff P. GABAA receptors: structure and function in the basal ganglia In: Tepper JM, Abercrombie ED, Bolam JP, editors. *Progress in Brain Research.* Amsterdam: Elsevier (2007). p. 21–41. doi: 10.1016/S0079-6123(06)60003-4
16. Nicoll RA, Malenka RC, Kauer JA. Functional comparison of neurotransmitter receptor subtypes in mammalian central nervous system. *Physiol Rev.* (1990) 70:513–65. doi: 10.1152/physrev.1990.70.2.513
17. Everington EA, Gibbard AG, Swinny JD, Seifi M. Molecular characterization of GABA-A receptor subunit diversity within major peripheral organs and their plasticity in response to early life psychosocial stress. *Front Mol Neurosci.* (2018) 11:18. doi: 10.3389/fnmol.2018.00018
18. El Idrissi A, Ding XH, Scalia J, Trenkner E, Brown WT, Dobkin C. Decreased GABA(A) receptor expression in the seizure-prone fragile X mouse. *Neurosci Lett.* (2005) 377:141–6. doi: 10.1016/j.neulet.2004.11.087
19. D'Hulst C, De Geest N, Reeve SP, Van Dam D, De Deyn PP, Hassan BA, et al. Decreased expression of the GABAA receptor in fragile X syndrome. *Brain Res.* (2006) 1121:238–45. doi: 10.1016/j.brainres.2006.08.115
20. D'Hulst C, Heulens I, Brouwer JR, Willemsen R, De Geest N, Reeve SP, et al. Expression of the GABAergic system in animal models for fragile X syndrome and fragile X associated tremor/ataxia syndrome (FXTAS). *Brain Res.* (2009) 1253:176–83. doi: 10.1016/j.brainres.2008.11.075
21. Kang JY, Chadchankar J, Vien TN, Mighdoll MI, Hyde TM, Mather RJ, et al. Deficits in the activity of presynaptic γ-aminobutyric acid type B receptors

contribute to altered neuronal excitability in fragile X syndrome. *J Biol Chem.* (2017) 292:6621–32. doi: 10.1074/jbc.M116.772541
22. D'Hulst C, Heulens I, Van der Aa N, Goffin K, Koole M, Porke K, et al. Positron emission tomography (PET) quantification of GABAA receptors in the brain of fragile X patients. *PLoS ONE.* (2015) 10:e0131486. doi: 10.1371/journal.pone.0131486
23. Olmos-Serrano JL, Paluszkiewicz SM, Martin BS, Kaufmann WE, Corbin JG, Huntsman MM. Defective GABAergic neurotransmission and pharmacological rescue of neuronal hyperexcitability in the amygdala in a mouse model of fragile X syndrome. *J Neurosci.* (2010) 30:9929–38. doi: 10.1523/JNEUROSCI.1714-10.2010
24. Berry-Kravis EM, Hessl D, Rathmell B, Zarevics P, Cherubini M, Walton-Bowen K, et al. Effects of STX209 (arbaclofen) on neurobehavioral function in children and adults with fragile X syndrome: a randomized, controlled, phase 2 trial. *Sci Transl Med.* (2012) 4:152ra127. doi: 10.1126/scitranslmed.3004214
25. Ligsay A, Van Dijck A, Nguyen DV, Lozano R, Chen Y, Bickel ES, et al. A randomized double-blind, placebo-controlled trial of ganaxolone in children and adolescents with fragile X syndrome. *J Neurodev Disord.* (2017) 9:26. doi: 10.1186/s11689-017-9207-8
26. Henderson C, Wijetunge L, Kinoshita MN, Shumway M, Hammond RS, Postma FR, et al. Reversal of disease-related pathologies in the fragile X mouse model by selective activation of GABAB receptors with arbaclofen. *Sci Transl Med.* (2012) 4:152ra128. doi: 10.1126/scitranslmed.3004218
27. Heulens I, D'Hulst C, Van Dam D, De Deyn PP, Kooy RF. Pharmacological treatment of fragile X syndrome with GABAergic drugs in a knockout mouse model. *Behav Brain Res.* (2012) 229:244–9. doi: 10.1016/j.bbr.2012.01.031
28. Chen X, Jacobs G, de Kam M, Jaeger J, Lappalainen J, Maruff P, et al. The central nervous system effects of the partial GABA-Aalpha2,3 -selective receptor modulator AZD7325 in comparison with lorazepam in healthy males. *Br J Clin Pharmacol.* (2014) 78:1298–314. doi: 10.1111/bcp.12413
29. The Dutch-Belgian Fragile XC. Fmr1 knockout mice: a model to study fragile X mental retardation. The Dutch-Belgian Fragile X Consortium. *Cell.* (1994) 78:23–33. doi: 10.1016/0092-8674(94)90569-X
30. Mientjes EJ, Nieuwenhuizen I, Kirkpatrick L, Zu T, Hoogeveen-Westerveld M, Severijnen L, et al. The generation of a conditional Fmr1 knock out mouse model to study Fmrp function *in vivo*. *Neurobiol Dis.* (2006) 21:549–55. doi: 10.1016/j.nbd.2005.08.019
31. Hays SA, Huber KM, Gibson JR. Altered neocortical rhythmic activity states in Fmr1 KO mice are due to enhanced mGluR5 signaling and involve changes in excitatory circuitry. *J Neurosci.* (2011) 31:14223–34. doi: 10.1523/JNEUROSCI.3157-11.2011
32. White AR, Tiwari D, MacLeod MC, Danzer SC, Gross C. PI3K isoform-selective inhibition in neuron-specific PTEN-deficient mice rescues molecular defects and reduces epilepsy-associated phenotypes. *Neurobiol Dis.* (2020) 144:105026. doi: 10.1016/j.nbd.2020.105026
33. Tiwari D, Schaefer TL, Schroeder-Carter LM, Krzeski JC, Bunk AT, Parkins EV, et al. The potassium channel Kv4.2 regulates dendritic spine morphology, electroencephalographic characteristics and seizure susceptibility in mice. *Exp Neurol.* (2020) 334:113437. doi: 10.1016/j.expneurol.2020.113437
34. Tse K, Puttachary S, Beamer E, Sills GJ, Thippeswamy T. Advantages of repeated low dose against single high dose of kainate in C57BL/6J mouse model of status epilepticus: behavioral and electroencephalographic studies. *PLoS ONE.* (2014) 9:e96622. doi: 10.1371/journal.pone.0096622
35. Gross C, Raj N, Molinaro G, Allen AG, Whyte AJ, Gibson JR, et al. Selective role of the catalytic PI3K subunit p110beta in impaired higher order cognition in fragile X syndrome. *Cell Rep.* (2015) 11:681–8. doi: 10.1016/j.celrep.2015.03.065
36. Thomas AM, Bui N, Graham D, Perkins JR, Yuva-Paylor LA, Paylor R. Genetic reduction of group 1 metabotropic glutamate receptors alters select behaviors in a mouse model for fragile X syndrome. *Behav Brain Res.* (2011) 223:310–21. doi: 10.1016/j.bbr.2011.04.049
37. Musumeci SA, Bosco P, Calabrese G, Bakker C, De Sarro GB, Elia M, et al. Audiogenic seizures susceptibility in transgenic mice with fragile X syndrome. *Epilepsia.* (2000) 41:19–23. doi: 10.1111/j.1528-1157.2000.tb01499.x
38. Bouet V, Boulouard M, Toutain J, Divoux D, Bernaudin M, Schumann-Bard P, et al. The adhesive removal test: a sensitive method to assess sensorimotor deficits in mice. *Nature Protocols.* (2009) 4:1560–4. doi: 10.1038/nprot.2009.125
39. Colter BT, Garber HF, Fleming SM, Fowler JP, Harding GD, Hooven MK, et al. Ahr and Cyp1a2 genotypes both affect susceptibility to motor deficits following gestational and lactational exposure to polychlorinated biphenyls. *Neurotoxicology.* (2018) 65:125–34. doi: 10.1016/j.neuro.2018.01.008
40. Schaefer TL, Vorhees CV, Williams MT. Mouse plasmacytoma-expressed transcript 1 knock out induced 5-HT disruption results in a lack of cognitive deficits and an anxiety phenotype complicated by hypoactivity and defensiveness. *Neuroscience.* (2009) 164:1431–43. doi: 10.1016/j.neuroscience.2009.09.059
41. Schaefer TL, Lingrel JB, Moseley AE, Vorhees CV, Williams MT. Targeted mutations in the Na,K-ATPase alpha 2 isoform confer ouabain resistance and result in abnormal behavior in mice. *Synapse.* (2011) 65:520–31. doi: 10.1002/syn.20870
42. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. *Behav Brain Res.* (2001) 125:279–84. doi: 10.1016/S0166-4328(01)00297-2
43. Gibson JR, Bartley AF, Hays SA, Huber KM. Imbalance of neocortical excitation and inhibition and altered UP states reflect network hyperexcitability in the mouse model of fragile X syndrome. *J Neurophysiol.* (2008) 100:2615–26. doi: 10.1152/jn.90752.2008
44. Razak KA, Dominick KC, Erickson CA. Developmental studies in fragile X syndrome. *J Neurodev Disord.* (2020) 12:13. doi: 10.1186/s11689-020-09310-9
45. Lovelace JW, Ethell IM, Binder DK, Razak KA. Translation-relevant EEG phenotypes in a mouse model of fragile X syndrome. *Neurobiol Dis.* (2018) 115:39–48. doi: 10.1016/j.nbd.2018.03.012
46. Bagni C, Zukin RS. A synaptic perspective of fragile X syndrome and autism spectrum disorders. *Neuron.* (2019) 101:1070–88. doi: 10.1016/j.neuron.2019.02.041
47. Schaefer TL, Davenport MH, Grainger LM, Robinson CK, Earnheart AT, Stegman MS, et al. Acamprosate in a mouse model of fragile X syndrome: modulation of spontaneous cortical activity, ERK1/2 activation, locomotor behavior, and anxiety. *J Neurodev Disord.* (2017) 9:6. doi: 10.1186/s11689-017-9184-y
48. Deacon RMJ. Measuring motor coordination in mice. *J Visual Experi JoVE.* (2013) 75:e2609. doi: 10.3791/2609
49. Thomas A, Burant A, Bui N, Graham D, Yuva-Paylor LA, Paylor R. Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacology.* (2009) 204:361–73. doi: 10.1007/s00213-009-1466-y
50. Bhattacharya A, Kaphzan H, Alvarez-Dieppa AC, Murphy JP, Pierre P, Klann E. Genetic removal of p70 S6 kinase 1 corrects molecular, synaptic, and behavioral phenotypes in fragile X syndrome mice. *Neuron.* (2012) 76:325–37. doi: 10.1016/j.neuron.2012.07.022
51. Ennaceur A, Delacour J. A new one-trial test for neurobiological studies of memory in rats. 1: behavioral data. *Behav Brain Res.* (1988) 31:47–59. doi: 10.1016/0166-4328(88)90157-X
52. Frankland PW, Wang Y, Rosner B, Shimizu T, Balleine BW, Dykens EM, et al. Sensorimotor gating abnormalities in young males with fragile X syndrome and Fmr1-knockout mice. *Mol Psychiatry.* (2004) 9:417–25. doi: 10.1038/sj.mp.4001432
53. Hessl D, Berry-Kravis E, Cordeiro L, Yuhas J, Ornitz EM, Campbell A, et al. Prepulse inhibition in fragile X syndrome: feasibility, reliability, and implications for treatment. *Am J Med Genet B Neuropsychiatr Genet.* (2009) 150B:545–53. doi: 10.1002/ajmg.b.30858
54. Gross C, Berry-Kravis EM, Bassell GJ. Therapeutic strategies in fragile X syndrome: dysregulated mGluR signaling and beyond. *Neuropsychopharmacology.* (2012) 37:178–95. doi: 10.1038/npp.2011.137
55. Gray JD, Rubin TG, Hunter RG, McEwen BS. Hippocampal gene expression changes underlying stress sensitization and recovery. *Mol Psychiatry.* (2014) 19:1171–8. doi: 10.1038/mp.2013.175
56. Harmony T. The functional significance of delta oscillations in cognitive processing. *Front Integrat Neurosci.* (2013) 7:83. doi: 10.3389/fnint.2013.00083
57. Bartos M, Vida I, Jonas P. Synaptic mechanisms of synchronized gamma oscillations in inhibitory interneuron

networks. *Nat Rev Neurosci.* (2007) 8:45–56. doi: 10.1038/nrn2044
58. Dias R, Sheppard WF, Fradley RL, Garrett EM, Stanley JL, Tye SJ, et al. Evidence for a significant role of α3-containing GABAA receptors in mediating the anxiolytic effects of benzodiazepines. *J Neurosci.* (2005) 25:10682–8. doi: 10.1523/JNEUROSCI.1166-05.2005
59. Morris H, Dawson G, Reynolds D, Atack J, Stephens D. Both α2 and α3 GABAA receptor subtypes mediate the anxiolytic properties of benzodiazepine site ligands in the conditioned emotional response paradigm. *Eur J Neurosci.* (2006) 23:2495–504. doi: 10.1111/j.1460-9568.2006.04775.x
60. Egashira N, Abe M, Shirakawa A, Niki T, Mishima K, Iwasaki K, et al. Effects of mood stabilizers on marble-burying behavior in mice: involvement of GABAergic system. *Psychopharmacology.* (2013) 226:295–305. doi: 10.1007/s00213-012-2904-9
61. Kazdoba TM, Leach PT, Silverman JL, Crawley JN. Modeling fragile X syndrome in the Fmr1 knockout mouse. *Intractable Rare Dis Res.* (2014) 3:118–33. doi: 10.5582/irdr.2014.01024
62. Fritschy JM, Panzanelli P. GABAA receptors and plasticity of inhibitory neurotransmission in the central nervous system. *Eur J Neurosci.* (2014) 39:1845–65. doi: 10.1111/ejn.12534
63. Rewal M, Jurd R, Gill TM, He DY, Ron D, Janak PH. Alpha4-containing GABAA receptors in the nucleus accumbens mediate moderate intake of alcohol. *J Neurosci.* (2009) 29:543–49. doi: 10.1523/JNEUROSCI.3199-08.2009
64. Anstee QM, Knapp S, Maguire EP, Hosie AM, Thomas P, Mortensen M, et al. Mutations in the Gabrb1 gene promote alcohol consumption through increased tonic inhibition. *Nat Commun.* (2013) 4:2816. doi: 10.1038/ncomms3816
65. McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Atack JR, et al. Sedative but not anxiolytic properties of benzodiazepines are mediated by the GABA(A) receptor alpha1 subtype. *Nat Neurosci.* (2000) 3:587–92. doi: 10.1038/75761

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The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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The Phenotypic Profile Associated With the *FMR1* Premutation in Women: An Investigation of Clinical-Behavioral, Social-Cognitive, and Executive Abilities

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The *FMR1* gene in its premutation (PM) state has been linked to a range of clinical and subclinical phenotypes among *FMR1* PM carriers, including some subclinical traits associated with autism spectrum disorder (ASD). This study attempted to further characterize the phenotypic profile associated with the *FMR1* PM by studying a battery of assessments examining clinical-behavioral traits, social-cognitive, and executive abilities in women carrying the *FMR1* PM, and associations with *FMR1*-related variability. Participants included 152 female *FMR1* PM carriers and 75 female controls who were similar in age and IQ, and screened for neuromotor impairments or signs of fragile X-associated tremor/ataxia syndrome. The phenotypic battery included assessments of ASD-related personality and language (i.e., pragmatic) traits, symptoms of anxiety and depression, four different social-cognitive tasks that tapped the ability to read internal states and emotions based on different cues (e.g., facial expressions, biological motion, and complex social scenes), and a measure of executive function. Results revealed a complex phenotypic profile among the PM carrier group, where subtle differences were observed in pragmatic language, executive function, and social-cognitive tasks that involved evaluating basic emotions and trustworthiness. The PM carrier group also showed elevated rates of ASD-related personality traits. In contrast, PM carriers performed similarly to controls on social-cognitive tasks that involved reliance on faces and biological motion. The PM group did not differ from controls on self-reported depression or anxiety symptoms. Using latent profile analysis, we observed three distinct subgroups of PM carriers who varied considerably in their performance across tasks. Among PM carriers, CGG repeat length was a significant predictor of pragmatic language violations. Results suggest a nuanced phenotypic profile characterized by subtle differences in select clinical-behavioral, social-cognitive, and executive abilities associated with the *FMR1* PM in women.

Keywords: *FMR1* premutation, broad autism phenotype, latent profile analysis, executive function, social cognition

INTRODUCTION

The *FMR1* gene plays a critical role in the expression of a range of clinical phenotypes, including both neurodevelopmental and neurodegenerative disorders. Located in the *5′ untranslated region (5′ UTR)* on the long arm of the X chromosome, *FMR1* encodes the Fragile X Mental Retardation Protein (FMRP), which is highly expressed in the brain and plays a role in synaptic plasticity (1–4). A full mutation of the *FMR1* gene (>200 cytosine-guanine-guanine [CGG] trinucleotide repeats) causes methylation and subsequent silencing of the gene, inhibiting production of FMRP and causing fragile X syndrome (FXS), a rare condition (∼1 in 4,000 males and ∼1 in 8,000 females) that is the most common inherited cause of intellectual disability and monogenic cause of autism spectrum disorder (ASD) (5–7). The *FMR1* premutation (PM; 55–200 CGG repeats), occurs in roughly 1 in 150–250 women, and is less prevalent in men (∼1 in 430–460) (8–12). Though the PM was once believed to have limited phenotypic expression, a number of clinical and subclinical phenotypes have since been identified, including PM-specific disorders (i.e., fragile X-associated primary ovarian insufficiency [FXPOI], fragile X-associated tremor/ataxia syndrome [FXTAS], and fragile X-associated neuropsychiatric disorders [FXAND]) (4, 13, 14). *FMR1*-related molecular genetic variation has been associated with these phenotypes, including CGG repeat length, such as mid-range vulnerability (90–110 repeats), toxic gain-of-function production of mRNA, RAN translation, and FMRP variation (15–23). As such, detailed phenotypic characterization of the PM is important from a public health perspective, with potential to connect complex human traits to known genetic variation.

An important body of work has described a number of clinical-behavioral traits among carriers of the *FMR1* PM, including ASD and subclinical ASD-related traits (24–27), anxiety and depression (28–33), and differences in social cognition and executive function (EF) (34–38). Because most prior work has examined these phenotypes in separate study samples, a key remaining question concerns whether such phenotypes co-occur, together constituting a phenotypic profile associated with the *FMR1* PM. Examining the co-occurrence of key phenotypes within individuals can also help to address whether such features may interrelate causally. It could be, for instance, that personality traits previously reported in PM groups reflect underlying differences in social cognition or EF. This study attempted to build on prior work to address these gaps by studying a range of clinical-behavioral, social-cognitive, and executive phenotypes associated with the *FMR1* PM within a relatively large sample of female PM carriers and controls.

Consistent with the strong phenotypic overlap observed between FXS and ASD (where most individuals with FXS exhibit at least some ASD symptoms, and many meet full diagnostic criteria for ASD) (24, 39–42), a number of studies have documented elevated rates of ASD among PM carriers, particularly males (∼14%) (24, 25, 43). Subclinical ASD traits have also been noted in the PM more generally, including personality styles such as social reticence (28) and rigid and perfectionistic traits (27, 34). Collectively known as the broad autism phenotype (BAP), this constellation of subclinical personality and language traits mirror the central features of ASD and are thought to index genetic liability to the disorder as they are observed at higher rates among first degree relatives of individuals with ASD relative to the general population (44–46) and associated with increased polygenic burden for ASD (47).

Losh et al. (27) evaluated personality and pragmatic language features of the BAP in PM carriers. Using direct assessment measures, including the Modified Personality Assessment Scale (MPAS) (48) and Pragmatic Rating Scale (PRS) (49), they found similar profiles of BAP personality traits and increased pragmatic language violations in PM carriers and mothers of individuals with ASD compared to controls. Further, within-family associations were detected in the PM group, showing that children with FXS whose mothers exhibited BAP traits had more severe ASD symptoms. Such co-segregation of ASD-related phenotypes within a subgroup of families is intriguing, particularly when considering known interactions between a number of ASD risk genes and the *FMR1* gene that might underlie these phenotypes (50, 51).

Beyond ASD-related risk, female PM carriers may display elevated symptoms of anxiety and depression, though rates vary considerably between studies (30, 32, 33, 52, 53). For instance, whereas Jiraanont et al. (53) reported higher diagnostic rates of depression (50%) and anxiety (33%) compared to controls (8.3 vs. 4.2%, respectively), Gossett et al. (52) reported no significant differences, but noted that the majority of the control group (∼54%) had clinically elevated symptoms. Studies including younger PM carriers and adults without children have also reported elevated mood and anxiety disorders, suggesting that these symptoms may arise early on and are not merely related to the stress of parenting a child with FXS (25, 32). Interestingly, there is some evidence to suggest that mood and anxiety symptoms may co-occur with other phenotypes, such as FXPOI and EF deficits (e.g., working memory), among subgroups of PM carriers (54, 55), underscoring the importance of examining how these symptoms may co-occur with other phenotypes, such as social-cognitive deficits and ASD-related features.

Subtle differences in social cognition (i.e., understanding mental states and feelings essential for supporting fluent interpersonal interactions) have also been observed among PM carriers. Studies of male PM carriers reported lower social-cognitive performance, including decreased theory of mind (34, 56) and reduced social reward processing, which related to lower FMRP (37). Male PM carriers have been reported to exhibit reduced neural activation in the amygdala compared to controls when viewing faces with fearful expressions (35). Far less is known about the social-cognitive profiles of females and potential biological correlates. Klusek et al. (57) found that, compared to controls, female PM carriers displayed reduced visual attention to others' direct gaze, which can impact the ability to interpret others' intent. These results were hypothesized to reflect difficulty with interpreting ambiguous social information or with recognizing direct gaze as an important social cue.

Even subtly impaired social-cognitive abilities have been associated with increased pragmatic language violations in ASD and FXS and the *FMR1* PM (58–62). For example,

subgroups of parents of individuals with ASD who displayed reduced social-cognitive skills tended to show elevated pragmatic language violations during semi-structured conversation (60). In FXS, increased pragmatic language deficits have been reported to cosegregate with more severe impairments in social cognition (62), and some evidence suggests that differences in social cognition among PM carriers may also relate to pragmatic language. In a study of visual attention, Winston et al. (63) reported atypical visual scanning patterns of faces among female PM carriers, but found that these differences were associated with *better* social cognition and pragmatic language, perhaps suggesting that some female PM carriers may employ alternative strategies for deducing meaningful information in social exchanges. Such findings suggest that subgroups of female PM carriers may exhibit social-cognitive or attentional patterns that facilitate pragmatic language, whereas for others, these domains may not be related. Consistent with this possibility, Winston et al. (64) identified a subgroup of PM carriers who demonstrated social viewing patterns characteristic of those found among parents of individuals with ASD and greater co-occurring pragmatic difficulties, and also had children with more severe ASD symptoms.

Numerous studies have also identified EF differences among female PM carriers without FXTAS (36, 65–67), including differences in working memory across both visual and verbal modalities (68, 69), inhibition (23, 54, 70), and attention (71). PM carriers also exhibit differences in language domains that are thought to reflect underlying EF difficulties, such as verbal disfluencies (72–74). Additionally, a recent study reported inefficient language processing and eye-voice coordination on a task of rapid naming of familiar objects; difficulties were particularly evident during the latter portions of the task when executive demands have been shown to be the greatest (22). The overlap between these language tasks and EF could suggest that aspects of the PM phenotype, such as subtle differences in pragmatic language, could cluster together with EF difficulties. For instance, Kraan et al. (54) found that EF deficits co-occurred with neuropsychiatric symptoms (such as depression and anxiety) in female PM carriers, though far less is known about the co-occurrence of executive dysfunction and other clinical-behavioral or cognitive correlates.

The present study aimed to further characterize the phenotypic spectrum associated with the *FMR1* PM by examining performance across a battery of clinical-behavioral, social-cognitive, and EF tasks in females with the *FMR1* PM in comparison to controls. Further, we utilized latent profile analyses to examine whether homogenous phenotypic subgroups within the PM might be identified based on distinct constellations of these phenotypes. Finally, following prior evidence that *FMR1*-related molecular-genetic variation is often associated with clinical-behavioral and cognitive phenotypes in the PM (21, 23, 30, 33, 37, 56, 75–77), we examined associations between the phenotypic battery and CGG repeat length and FMRP.

TABLE 1 | Participant characteristics.

	Premutation carriers *M* (*SD*)	Female controls *M* (*SD*)
N	152	75
Chronological age	44.43 (8.76)	42.50 (9.21)
IQ	112.90 (9.78)	114.67 (11.20)
VIQ	110.69 (10.59)	110.29 (12.20)
PIQ	**111.97 (9.58)**	**113.45 (13.52)**

VIQ, Verbal IQ; PIQ, Performance IQ.
Participants did not significantly differ on age, IQ, or VIQ (ps > 0.230), but did differ on PIQ (p = 0.039; in bold).

METHODS

Participants

Participants included 152 female *FMR1* PM carriers and 75 adult female controls. Only females were included in this study to control for biological sex, and to ensure feasible ascertainment. All participants were native English speakers because of the language-based nature of many of the tasks. PM carriers were recruited from genetic clinics, physicians' offices, advocacy groups, and the Research Participant Registry Core of the Carolina Institute for Developmental Disabilities at the University of North Carolina at Chapel Hill. PM status was confirmed by genetic testing (direct or confirmation by prior medical records). Controls were recruited through community resources (e.g., schools and child care centers, local community events) and word of mouth, as well as through the Communication Research Registry at Northwestern University. Controls were recruited as part of larger family genetics studies of ASD/BAP and *FMR1*-related conditions, and were therefore screened for personal or family history of FXS, ASD, and genetically-based conditions associated with ASD. **Table 1** summarizes participant characteristics. Groups did not differ significantly in age ($p = 0.233$), or Full-Scale IQ (FSIQ; $p = 0.250$). In the PM sample, 135 participants had at least one child with FXS. Nine participants were mothers of children without FXS, and 16 were not mothers.

Potential participants were asked to report any prior diagnosis of FXTAS or Parkinsonism, and were excluded if they endorsed such symptoms. Additionally, participants completed a reduced set of screening questions from the FXTAS Rating Scale (78, 79), assessing action or postural tremor, standing capacities, tandem gait, and handwriting-related items. Four individuals were excluded for rating positive on one or both of these indices.

All study procedures were approved by the Institutional Review Boards of participating universities. All participants provided informed consent to participate.

Clinical-Behavioral Measures

Assessment of the BAP

Modified Personality Assessment Schedule

The Modified Personality Assessment Schedule (MPAS) (48) was used to assess three core personality traits associated with the BAP (i.e., social aloofness, rigidity, and untactfulness) among the

PM carrier group. This instrument has been used extensively in family studies of ASD [e.g., (44, 45)], and consists of a direct-assessment clinical interview designed to elicit examples regarding the endorsement of each personality trait. Traits are rated based on concrete behavioral examples using a 3-point scale (0 = absent, 1 = partially present, and 2 = present). Given that the BAP occurs at relatively low rates in individuals without a family history of ASD (46, 80), and the overarching goal of assessing how BAP personality traits might relate to other phenotypes among PM carriers, controls were not administered the MPAS.

Each interview was coded by two independent raters who were trained to at least 80% reliability, and were blind to group status, with final scores determined through consensus. Individuals were rated BAP (+) for social features if they had a score of 2 on either social aloofness or untactfulness, and BAP (+) for rigid features if they had a score of 2 in the rigid domain. Finally, individuals were rated BAP (–) if they scored either a 0 or 1 on all domains. Average reliability prior to consensus coding was 76.28%.

Pragmatic Rating Scale

Twenty-minute semi-structured conversations were conducted between examiners and participants concerning their "life history" [for detailed description, see (27)]. Participants were asked about a series of topics that pertained to early childhood and friendships, current employment, hobbies, and romantic relationships. To elicit specific pragmatic behaviors during the conversation, such as reciprocity and the ability to clarify a message, examiners were trained to periodically offer related personal information and feign confusion. Conversations were coded from video by two trained research assistants, who were blind to participant family diagnosis, using the Pragmatic Rating Scale (PRS) (49). The PRS captures pragmatic language features of the BAP, and assesses 26 different pragmatic skills (e.g., providing adequate detail and background information), which are rated on a three-point scale from 0 (not present), 1 (somewhat present), to 2 (definitely present). In addition to a total number of pragmatic language violations, scores on three factor scores [dominant, withdrawn, and suprasegmental factors; see (27)] were also examined. All files were consensus coded for a best estimate rating used in analyses. Reliability prior to consensus coding was 84.07%.

Mood and Anxiety

Beck Depression Inventory-II

Depression symptoms were assessed using the Beck Depression Inventory-II (BDI-II) (81). The BDI-II is a 21-item measure of depressive symptoms on a 4-point scale, and has been normed in both typical and clinical populations. It follows the Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition (DSM-IV) criteria for depression and assesses the presence and severity of depression with high reliability (Coefficient Alpha = 0.92). Scores on this measure range from 0 to 63; scores falling in the 0–13 range suggest the presence of minimal depressive symptoms, 14–19 indicate mild, 20–28 moderate, and 29–63 severe.

State-Trait Anxiety Inventory

The State-Trait Anxiety Inventory (STAI) (82) assessed anxiety symptoms. Used extensively in psychiatric research and practice, the STAI consists of two 20-item questionnaires that evaluate current (i.e., state) and more persistent (i.e., trait) symptoms of anxiety. The STAI provides a continuous measure of anxiety, with a range for each subtest of 20–80, with higher scores indicating greater anxiety (83, 84). Clinically significant anxiety has been suggested for scores at or above 39 (83, 84). Standard scores were used in all analyses.

Mini-International Neuropsychiatric Interview

The Mini-International Neuropsychiatric Interview (MINI) (85) is a well-validated and widely used structured psychiatric diagnostic interview instrument to evaluate current and past depressive episodes along DSM-IV criteria. This measure is intended as a tool for dichotomous categorization (i.e., yes/no) of psychiatric symptoms of depression and anxiety. Two-sided Fisher's exact tests were used to compare rates of symptoms across groups.

Social Cognition

Reading the Mind From the Eyes Task

The Eyes Task (86) was used to index the ability to infer psychological states from viewing the eye region of the face. Participants were shown 36 different images of eyes expressing different psychological states and were asked to select a corresponding term from an array of four words. Performance was measured as the proportion of correct responses.

Trustworthiness of Faces Task

The Trustworthiness of Faces Task (87, 88) assessed the ability to use facial expressions to infer the social attribute of trustworthiness. Participants were asked to rate the trustworthiness of a series of 42 faces, which varied in gender, expression, and gaze. Ratings were made on a seven-point scale (−3 to +3), with negative scores denoting less trustworthiness, a score of "0" denoting neutral trustworthiness, and positive scores indicating greater trustworthiness. Faces were categorized into "negatively valenced" and "positively valenced" based on valence ratings from the original control group (87).

The Movie Stills Task

The Movie Stills Task (89) measured the extent to which individuals use facial information to infer the emotional content of a scene. Participants were asked to determine the emotional state (happy, sad, afraid, angry, surprised, disgusted, or neutral) from a series of 16 movie stills. The first trial obscured the faces of the characters in the image, and the second trial presented the image with the faces intact. Following prior work (89), scores were derived from the control participants ($n = 49$). For each of the 16 images with faces, the proportion of controls who selected a given emotion was first calculated to determine the distribution of responses. Scores were then weighted based on the distribution of responses, and parametrically transformed to give partial credit to alternative responses. For instance, if on a given image, 50% of the controls chose angry, 40% chose afraid,

and 10% chose neutral, angry was given a score of 1, afraid was weighted as 0.8, and a neutral response was weighted as 0.2, whereas all other responses received no credit. For each image, the emotion that was the most often chosen by the controls was determined as the target emotion. Scores were then averaged for each emotion condition.

Point Light Tasks

The ability to recognize emotions through biological motion was assessed using a task where participants viewed light emitting diodes affixed to an actor's body, as the figure moved through a black space in different manners (90). Participants completed two versions of this task—one in which they judged basic emotions (e.g., happy, angry) and another where they judged the trustworthiness conveyed by the body movements. As with the Movie Stills task, each emotion was given a weighted score based on the proportion of controls who selected each emotion per trial. The weighted scores were used to calculate emotional accuracy.

During the second version of the task, participants were asked to rate the trustworthiness of each display based on its pattern of movement. Trustworthiness was rated on a scale of 1–5, with 1 representing the most trustworthy and 5 representing the least trustworthy. For analyses and interpretation of this version, images were divided into "lower" and "higher" trustworthiness based on original control ratings. Raw ratings of trustworthiness were used in analyses.

Executive Function

Behavior Rating Inventory of Executive Function—Adult Version

Participants completed the Behavior Rating Inventory of Executive Function-Adult Version (BRIEF-A) (91), a 75-item self-report questionnaire that assesses multiple domains of executive functioning. Participants had the option to complete this self-report questionnaire in the lab, or remotely *via* an online link to the questionnaire. Each item was rated on a three-point Likert scale (i.e., never, sometimes, often), indicating the extent to which a behavior occurred over the past 6 months**.** Raw scores were converted into standardized t-scores across nine domains: Inhibition, Shift, Emotional Control, Self-Monitor, Initiate, Working Memory, Plan/Organize, Task Monitor, and Organization of Materials. Higher scores indicate poorer executive functioning abilities. Scores from each domain yield an overall Global Executive Composite (GEC) score, and two composite scores, Behavioral Regulation Index (BRI) and Metacognitive Index (MI). *T*-scores 65 or greater indicate clinically significant deficits in executive functioning.

FMR1 Molecular-Genetic Variation

Polymerase chain reaction and Southern blot techniques were used to confirm PM status and determine CGG repeat length. FMRP was assayed in lymphocytes isolated from blood, using a Luminex Assay to reliably quantify levels of FMRP (92). Activation ratio (AR) measures the proportion of cells carrying the normal allele on the active X chromosome (78), and was determined by the ratio of the intensity of the normal *FMR1* unmethylated band divided by the sum of the intensities of the normal unmethylated and methylated bands (93). **Table 2** presents descriptive information regarding *FMR1*-related variation.

TABLE 2 | *FMR1* characteristics in PM carriers.

	***M* (*SD*)**	**Range**
CGG repeat length	89.68 (16.23)	59–126
Quantitative FMRP	0.02 (0.01)	0.00–0.04
Activation ratio	0.49 (0.23)	0.00–0.95

CGG, Cytosine-Guanine-Guanine; FMRP, fragile X mental retardation protein (pg/ug). Activation ratio reflects the percentage of cells for which the active X chromosome contains an unaffected FMR1 gene.

Data Analysis

All variables were examined for normality of distribution. General linear models (i.e., analyses of variance, ANOVAs) were used to assess group differences across clinical-behavioral, social-cognitive, and EF measures. Effect sizes for correlational analyses were interpreted as small (0.20), medium (0.50), and large (0.80) (94). Effect sizes for ANOVAs are reflected as partial eta squared (η_p^2), and were interpreted as small (0.01), medium (0.06), and large (0.14) (94). Bonferroni corrections were applied to adjust for multiple comparisons. Because groups did not differ significantly on age or IQ ($p > 0.153$), and age and IQ were generally not associated with variables of interest, we did not control for these variables in analyses.

To examine whether there may be subgroups who display specific patterns of performance across tasks, we conducted latent profile analyses (LPA) in the PM group. LPA serves to identify latent subpopulations having different configural profiles based on variables hypothesized to comprise meaningful phenotypes (95). A total of 14 variables reflecting performance across measures were included as numerical indicators in the LPA (see **Table 3**). Given numerical scale differences between measures, all variables were *z*-scored to improve interpretability.

Using an iterative process, we evaluated LPA solutions ranging from one to six potential PM profiles using the following fit measures to determine the best solution: the Akaike Information Criterion (AIC), the Bayesian Information Criterion (BIC), Entropy, a Bootstrap Likelihood Ratio Test (BLRT), and a Sattora–Bentler Scaled likelihood ratio chi-square difference test (TRd) (95–98). We also considered the theoretical interpretability of the profiles and profile size (for technical details on LPA, see **Supplementary Materials**). Profile subgroups were examined in follow-up analyses to evaluate any meaningful differences in age, IQ, *FMR1*-related genetic variation (i.e., CGG repeat length, AR, and quantitative FMRP), maternal status, or severity of ASD symptoms in their children (measured in oldest child in multiplex families using the ADOS-2) (99).

Associations between clinical-behavioral and cognitive phenotypes with molecular-genetic variables (i.e., *FMR1* CGG repeat length, FMRP) were conducted in the PM group only using Pearson correlations. Subsequently, linear regression

TABLE 3 | Measures included as numerical indicators in latent profile analysis (LPA).

Domain	Measures	Variables
Personality Features of the BAP	Modified Personality Assessment Schedule—Revised (MPAS)	1. MPAS-Social (social aloofness + untactful) 2. MPAS-Rigid
Pragmatic Language Features of the BAP	Pragmatic Rating Scale (PRS)	1. PRS-Dominating Conversation factor score 2. PRS-Withdrawn factor score 3. PRS-Suprasegmentals factor score
Executive Functioning	Brief Rating Inventory of Executive Function (BRIEF)	1. BRIEF—Global Executive Composite *T*-score
Mood and Anxiety	Beck Depression Inventory (BDI) The State-Trait Anxiety Inventory (STAI)	1. BDI total score 2. State Anxiety subscale total score 3. Trait Anxiety subscale total score
Social Cognition	Reading the Mind in the Eyes Task Trustworthiness of Faces Task Movie Stills Task Point Light Basic Task	1. Eyes Task percent correct 2. Trustworthiness mean ratings 3. Movie skills with faces mean emotion ratings 4. Movie Stills without faces mean emotion ratings 5. Point Light mean emotion ratings

All variables were z-scored prior to inclusion in the LPA. BAP, Broad autism phenotype.

models were applied to examine the extent to which molecular-genetic variables might predict phenotypic outcomes. For regression models that included CGG repeat length, separate models were conducted using linear and curvilinear CGG terms (i.e., CGG squared) given evidence of varied associations between CGG repeat length and behavioral measures in the PM (20, 21, 33). Given the presence of two X chromosomes in females, in models that included CGG repeat length, AR and an interaction term (i.e., CGG × AR) were included as covariates.

RESULTS

Characterization and Group Differences on Clinical-Behavioral and Cognitive Measures

BAP

MPAS

Among those with MPAS data in the PM group ($n = 87$), 54% of the sample was characterized as BAP (+). Thirty-two percent of the sample exhibited either social or rigid BAP personality features ($ns = 28$), and 10% of the sample ($n = 9$) displayed both social and rigid BAP personality features. Prior studies using the MPAS indicate ∼10–15% of controls display any BAP personality features (27, 46).

Pragmatic Rating Scale

Females with the PM exhibited significantly higher total PRS scores (i.e., more violations) than the control group [$F_{(1,185)} = 5.65$, $p = 0.019$, and $\eta_p^2 = 0.03$]. Examining factor scores on the PRS revealed that the PM group demonstrated a more dominant conversational style than controls [$F_{(1,174)} = 7.67$, $p = 0.006$, and $\eta_p^2 = 0.04$], but did not differ from controls in their withdrawn [$F_{(1,174)} = 0.60$, $p = 0.439$, and $\eta_p^2 = 0.00$] or suprasegmental scores [$F_{(1,174)} = 2.08$, $p = 0.151$, $\eta_p^2 = 0.01$, see **Figure 1**].

Mood and Anxiety

Beck Depression Inventory

PM carriers did not significantly differ from controls on BDI-II total scores [$F_{(1,\ 69)} = 2.58$, $p = 0.113$, $\eta_p^2 = 0.04$].

State Trait Anxiety Inventory

PM carriers endorsed marginally more Trait and State anxiety than controls [$F_{(1\ 77)} = 3.72$, $p = 0.058$, $\eta_p^2 = 0.05$; $F_{(1,\ 76)} = 3.02$, $p = 0.086$, $\eta_p^2 = 0.04$, respectively]. Clinically significant symptoms of anxiety (>39) were self-reported in 94.7% of PM carriers and 90% of controls.

MINI

PM carriers and controls did not differ on rates of current generalized anxiety disorder [22 vs. 14%, $X^2_{(1,144)} = 1.40$, $p = 0.285$], past generalized anxiety disorder [19.6 vs. 11.9%, $X^2_{(1,144)} = 1.23$, $p = 0.338$], current major depressive disorder [5 vs. 0%, $X^2_{(1,144)} = 2.75$, $p = 0.164$], or past major depressive disorder [25.7 vs. 19.6%, $X^2_{(1,144)} = 0.75$, $p = 0.440$].

Social Cognition

Reading the Mind in the Eyes

PM carriers had marginally lower scores on the Eyes Task than controls [$F_{(1,203)} = 1.49$ $p = 0.075$, $\eta_p^2 = 0.02$].

Movie Stills

PM carriers demonstrated significantly lower overall performance on movie stills with faces [$F_{(1,\ 23)} = 6.03$, $p = 0.015$, $\eta_p^2 = 0.03$], but did not differ from controls in performance on stimuli without faces ($p = 0.389$). PM carriers had marginally lower scores on neutral and sad stimuli with faces ($p = 0.074$ and 0.069, respectively).

Point Light Basic

PM carriers scored significantly lower than controls on happy stimuli [$F_{(1,\ 97)} = 4.25$, $p = 0.042$, $\eta_p^2 = 0.04$], but did not differ in overall performance ($p = 0.256$) or on other emotion types ($p > 0.145$).

Point Light Trustworthiness

No differences emerged between groups in overall point light trustworthiness scores [$F_{(1,\ 24)} = 0.94$, $p = 0.335$, $\eta_p^2 = 0.008$], or for more trustworthy or less trustworthy stimuli [$F_{(1,\ 24)} = 0.74$, $p = 0.392$, $\eta_p^2 = 0.006$; $F_{(1,\ 24)} = 0.65$, $p = 0.422$, $\eta_p^2 = 0.005$, respectively].

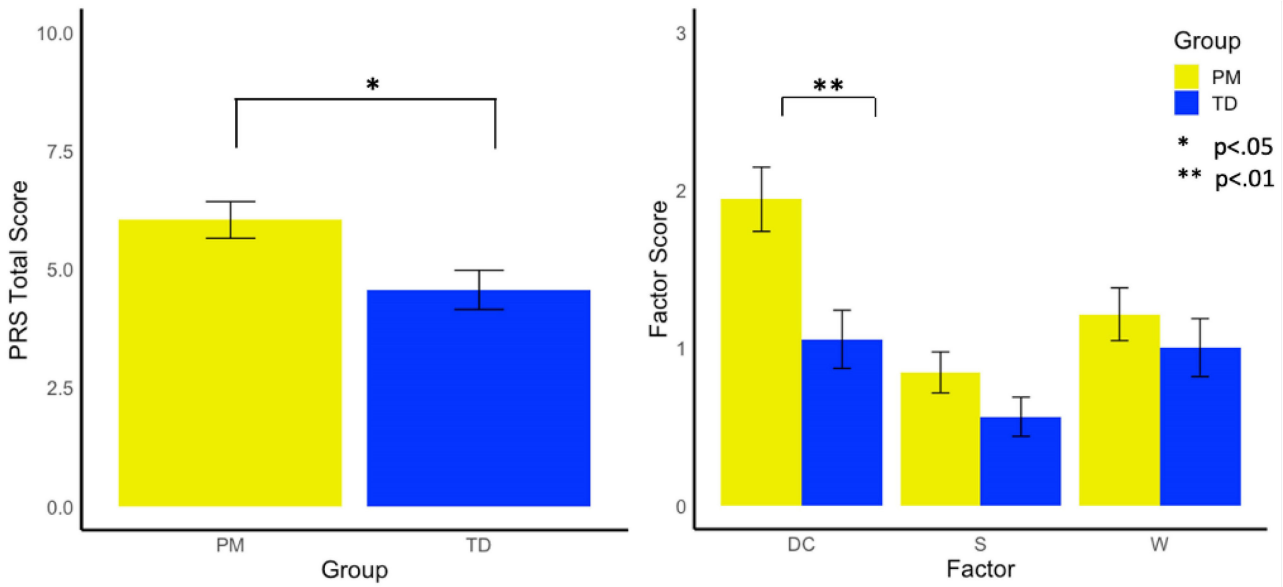

FIGURE 1 | Group differences in pragmatic rating scale total scores and factors. DC, Dominates Conversation Factor; S, Suprasegmental Factor; W, Withdrawn Factor.

Trustworthiness of Faces

PM carriers rated faces as significantly less trustworthy than controls overall [$F_{(1, 147)} = 9.31$, $p = 0.003$, $\eta_p^2 = 0.06$]; differences were evident on both positive and negative valanced faces [$F_{(1, 147)} = 9.80$, $p = 0.002$, $\eta_p^2 = 0.06$; $F_{(1,147)} = 7.74$, $p = 0.006$, $\eta_p^2 = 0.05$, respectively; see **Figure 2**].

Executive Function

BRIEF-A

Twenty-nine percent of PM carriers exceeded clinical cut-off on the GEC of the BRIEF-A, whereas only one female control exceeded clinical cut-off. PM carriers scored significantly higher (i.e., greater EF difficulty) than controls on the GEC, BRI, and MI scales [$F_{(1, 58)} = 6.55$, $p = 0.013$, $\eta_p^2 = 0.10$; $F_{(1, 58)} = 4.47$, $p = 0.039$, $\eta_p^2 = 0.07$; $F_{(1, 58)} = 6.81$, $p = 0.012$, $\eta_p^2 = 0.12$; see **Figure 3**].

Latent Profile Analysis of PM Carriers

LPA solutions for up to six models are presented in **Table 4**, and a three-profile solution was selected for interpretability.

The results of the three-profile solution are presented in **Figure 4**. The first profile (Profile 1) contained 77.6% of PM carriers ($n = 118$, average posterior probability = 0.983), and included individuals whose scores across domains consistently fell around the PM group mean, with limited variation across domains. The second profile (Profile 2) contained 17% of PM carriers ($n = 26$, average posterior probability = 0.871) and reflected individuals with increased mood and anxiety symptoms, slightly elevated social and rigid personality features of the BAP, and increased pragmatic language violations in the suprasegmental domain (e.g., intonation of voice, rate of speech, and volume modulation) as compared to other PM carriers. The final profile (Profile 3) contained 5.3% of PM carriers ($n = 8$, average posterior probability = 0.959) and included individuals who demonstrated elevated executive dysfunction, poorer social-cognitive abilities across tasks, elevated social and rigid personality features of the BAP, and increased pragmatic language violations in the listener expectation domain (e.g., unable to clarify, failure to reciprocate) relative to other PM carriers.

Follow up comparisons showed no significant differences across the profile groups in age, IQ, *FMR1*-related genetic variation, maternal status, or presence of ASD diagnosis or severity of ASD symptoms in their children (all $ps > 0.200$).

Multiple Regressions: PM Phenotypes and *FMR1*-Related Variation

A linear regression model including CGG repeat length predicted a significant amount of variance in PRS scores ($R^2 = 0.09$, $p = 0.046$; see **Table 5**). No other associations were observed between CGG repeat length and other clinical-behavioral, social-cognitive, or EF measures ($p > 0.169$).

FMRP was a significant predictor of performance on one social-cognitive task: Movie Stills (Happy) with faces. Specifically, quantitative FMRP predicted 12% of the variance in Movie Stills (Happy) with faces ($b = -0.06$, $p = 0.005$). Quantitative FMRP levels were not significantly associated with other clinical-behavioral, social-cognitive, or EF measures ($p > 0.131$).

DISCUSSION

This study characterized clinical-behavioral, social-cognitive, and EF features in females with the *FMR1* PM. Consistent with

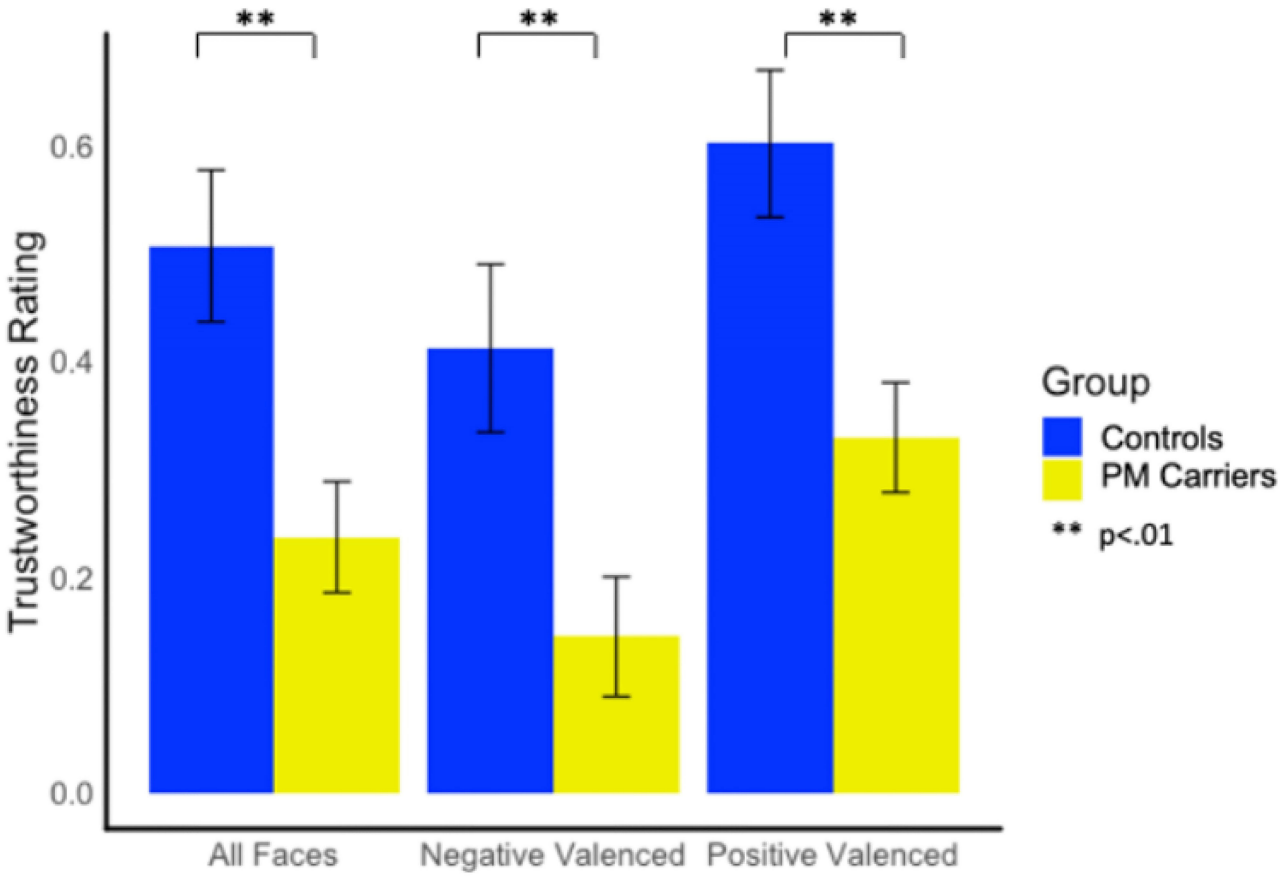

FIGURE 2 | Group differences in trustworthiness ratings.

prior work, results revealed elevated rates of subclinical ASD-related personality and language features among women with the PM, as well as increased self-reported difficulties in executive functioning, but no differences in mood and anxiety symptoms compared to controls. Some differences in social-cognitive tasks were also observed, including differences in complex social-emotional judgements of trustworthiness of faces, and in accuracy identifying basic emotions when viewing complex scenes. Latent profile analysis revealed three subgroups within the PM group who exhibited distinct phenotypic profiles across clinical-behavioral, social-cognitive, and EF measures, which together with group differences, and some associations with *FMR1*-related variation, may provide insights into phenotypic profiles associated with the *FMR1* PM.

Replicating prior work, personality and pragmatic language differences that define the BAP were observed at elevated rates in the PM carrier group. Direct measurement tools, scored by raters blind to group status, identified approximately half of the PM carrier group as displaying personality traits consistent with the BAP, compared with published rates among individuals without a family history of ASD or FXS ranging from ∼5 to 10% [e.g., (46, 80)]. Pragmatic language differences have been repeatedly observed among PM carriers (27, 57, 100–102), and were evident in this study as well, with additional patterns noted across the types of pragmatic language violations occurring more frequently in the PM group, who tended to display a more dominant conversational style (e.g., overly detailed, tangential language) than controls. Differences in pragmatic language have consistently emerged as a phenotypic marker associated with the PM in females (27, 100–102), and among individuals with FXS, particularly those who meet criteria for ASD (62, 103–109) and may be of clinical importance. For instance, prior work has shown that pragmatic language violations among mothers of children with FXS were associated with reduced self-reported quality of life for mothers (102), as well as elevated ASD symptoms and weaker expressive and receptive language in their children (27, 100). Together, this suggests that pragmatic language may be relevant to the well-being of both mothers and their children.

In contrast to previous work demonstrating higher rates of mood and anxiety disorders in PM carriers (25, 28, 30), we found no significant differences in depression scores between PM carriers and controls and only marginal differences from controls for anxiety symptoms, with both groups self-reporting elevated

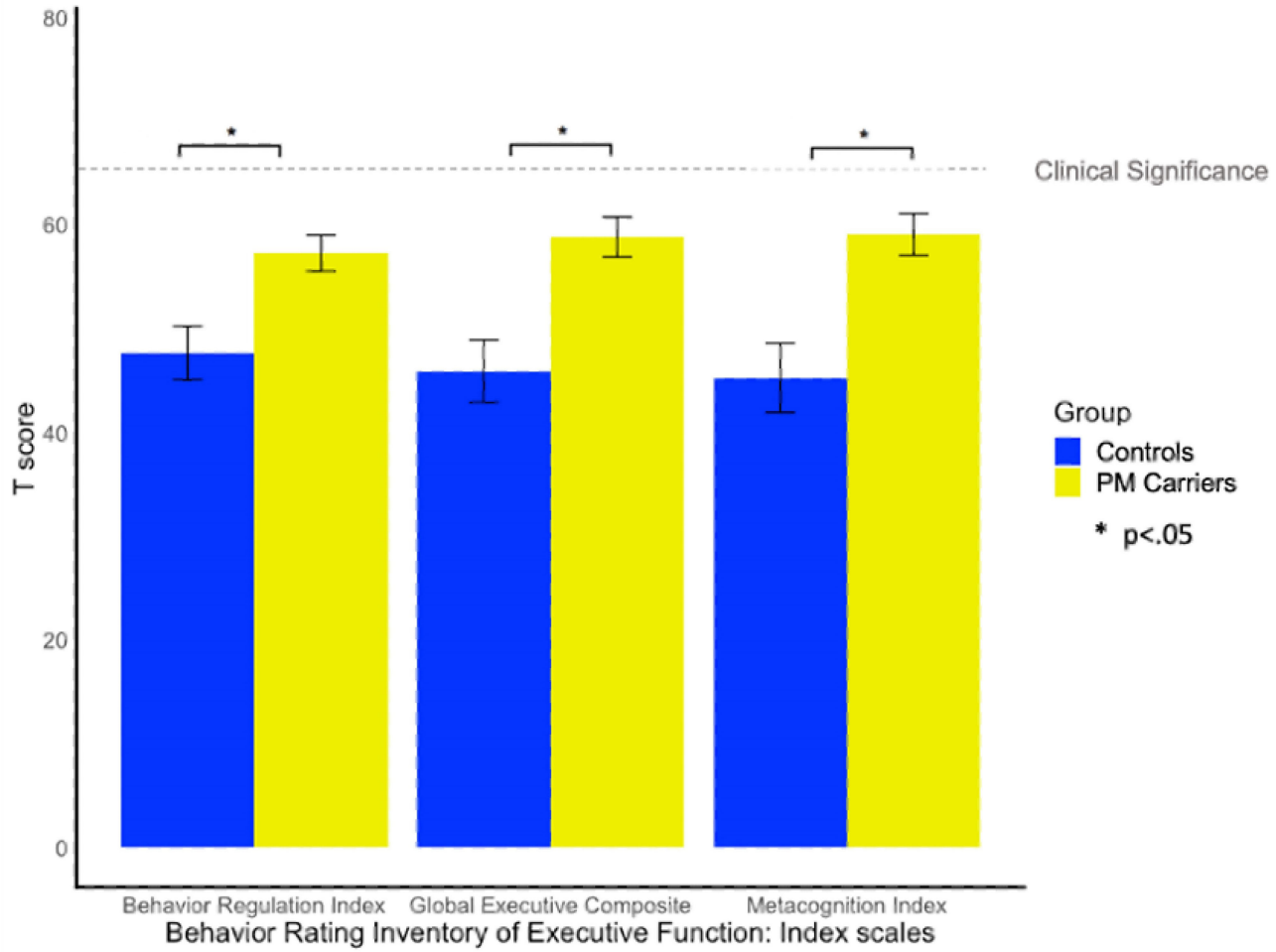

FIGURE 3 | Group differences on the BRIEF-A. BRIEF-A *T*-scores > 65 indicate clinically elevated difficulties relative to the standardization sample.

TABLE 4 | Indices of model fit for latent profile analysis.

Number of profiles	AIC	BIC	aBIC	Entropy	BLRT *p*-value	Log likelihood	TRd
1	3,782.24	3,866.90	3,778.29	–	–	−1,863.118	–
2	3,683.82	3,813.85	3,677.75	0.95	<0.001	−1,798.911	$\chi^2_{(15)} = 111.79, p < 0.001$
3	**3,621.14**	**3,796.52**	**3,612.95**	**0.91**	**<0.001**	**−1,752.569**	$\boldsymbol{\chi^2_{(15)} = 99.07, p < 0.001}$
4	3,573.16	3,793.90	3,562.86	0.76	<0.001	−1,713.578	$\chi^2_{(15)} = 61.46, p < 0.001$
5	3,546.71	3,812.82	3,534.30	0.78	<0.001	−1,685.357	$\chi^2_{(15)} = 54.79, p < 0.001$
6	3,520.57	3,832.03	3,506.03	0.79	<0.001	−1,657.284	$\chi^2_{(15)} = 56.66, p < 0.001$

AIC, Akaike Information Criterion; BIC, Bayesian Information Criterion; aBIC, Adjusted Bayesian Criterion; BLRT, Bootstrap Likelihood Ratio Test; TRd, Sattora–Bentler Scaled Likelihood Ratio Chi-square Difference Test. The 3-class solution (bolded) was selected for final inclusion.

anxiety symptoms (>90% of participants). Rates of anxiety and depression were far lower in both groups using the MINI, a standardized psychiatric interview (ranging from 0 to 25%), and also comparable to rates reported in prior work (31, 33, 110). In follow-up analyses, no differences were observed on depression and anxiety features according to maternal status, or the number of affected children in each family, suggesting these findings are not related to parenting factors.

Unique to the present study was the inclusion of multiple measures of social cognition within a single sample, permitting characterization of social cognitive strengths and weaknesses across different types of stimuli. Differences in social-cognitive profiles have been reported among PM carriers compared to controls, particularly among males (35, 37). This study identified key social-cognitive differences in female carriers of the PM in specific tasks, which echo findings observed among individuals

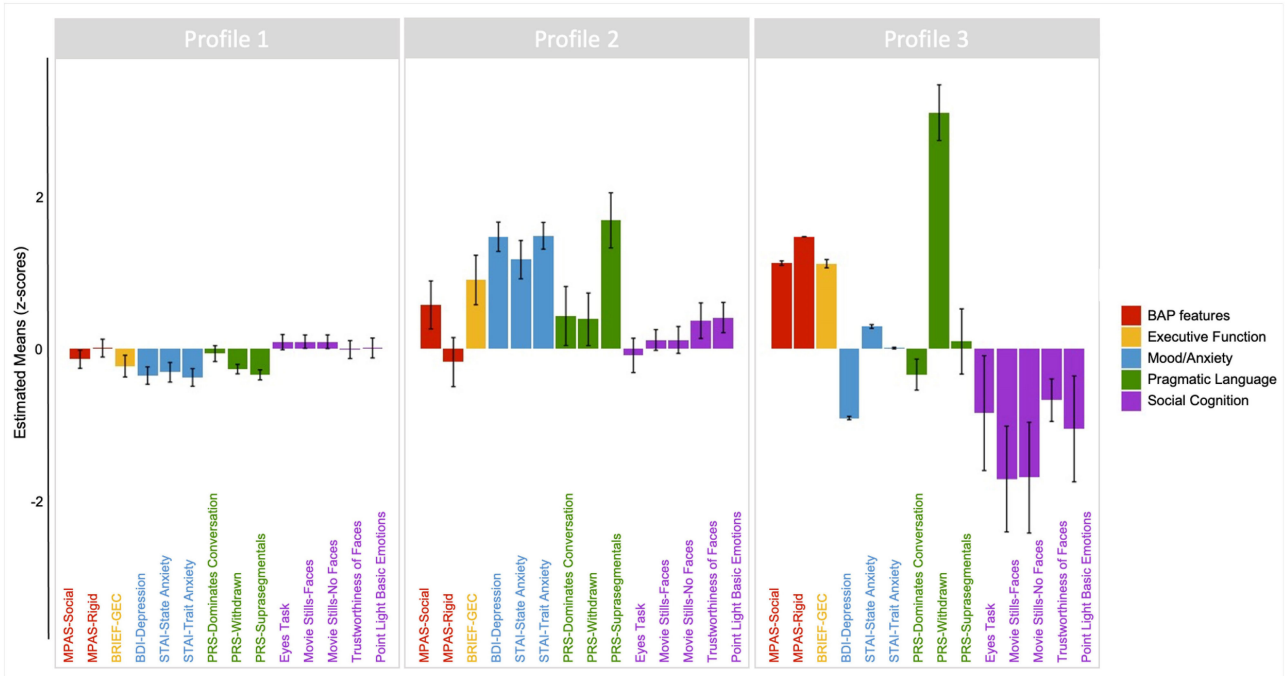

FIGURE 4 | Latent profile groups emerging from performance profiles across clinical-behavioral, pragmatic language, social cognitive, and executive tasks. Positive scores reflect poorer or more atypical performance along the following: BAP personality features (red); Executive function (yellow); Mood/anxiety (blue); Pragmatic language features of the BAP (green). For social cognition (purple), negative scores reflect poorer performance relative to the mean.

TABLE 5 | CGG repeat length predicts elevated pragmatic language violations (on Pragmatic Rating Scale).

	Unstandardized *b* coefficient	S.E.	β	*t*	*p*-value
CGG repeat length	**−0.21**	**0.10**	**−0.96**	**−2.05**	**0.046**
Activation Ratio	−25.43	13.24	−1.70	−1.92	0.062
CGG*Activation Ratio	0.30	0.15	2.12	1.96	0.057

All significant effects ($p < 0.05$) are noted in bold. CGG, Cytosine-Guanine-Guanine.

with FXS (62, 111). PM carriers differed in rating trustworthiness in response to faces ranging in emotional valence, a task that draws on social-cognitive and social decision-making skills. Differences on this task have been reported in ASD and the BAP in clinically unaffected relatives (61), and also among patients with bilateral amygdala damage, implicating this brain region in atypical performance in these groups (87, 89, 112). In studies of the PM, differences in amygdala volume and activation have been linked to aspects of social cognition among male carriers (35, 37), and these findings may suggest similar relationships among women with the PM that will be important to investigate in future work.

PM carriers were also less accurate in inferring emotions from faces when viewing still movie scenes. These results may indicate that PM carriers use different strategies in making social judgments relative to controls when viewing faces. Prior work that used eye tracking to examine looking patterns in response to faces indicates that PM carriers use different visual strategies from controls to inform social judgments (63). In the present study, marginal differences were also observed on the task involving reading complex thoughts and emotions from the eye region of the face. Thus, sensitivity to gaze and emotion expression, as measured by these social-cognitive tasks, may be an objective behavioral marker of underlying neural processes associated with interpreting emotion within the PM (35).

Consistent with prior reports of EF impairment associated with the PM (113), the PM group exhibited significantly higher executive functioning difficulty than controls, with approximately one third of the PM group reporting clinically significant EF difficulties. Characterization of the cognitive phenotype associated with the PM is especially important for understanding the manifestation of the neurodegenerative disorder, FXTAS, which may reveal subclinical phenotypic markers that are evident in a subgroup of PM carriers who go on to develop the disorder, as dysexecutive symptoms are a hallmark of FXTAS, particularly in males (114). Nonetheless, females with the PM exhibit differences in EF symptoms even without a diagnosis of FXTAS (68, 69, 115, 116). This study highlights the

value of using self-report measures of EF in studies of the PM, as prior work has typically used online behavioral measures [e.g., see (113) for review]. The use of the BRIEF-A among females with the PM has been limited to only a handful of prior studies (23, 117), but is useful in that clinical significance may be easily determined and a standardized self-report measure can enable cross-cohort comparisons. Of note, however, such self-report measures could lead to over-reporting of symptoms (118, 119), and so may best be interpreted within the context of results from studies employing direct assessment of executive skills.

Complementing results from group comparisons, latent profile analyses revealed three distinct groups of PM carriers who displayed different profiles of performance across the various domains. Profile 1 comprised the largest subgroup, and represented those scoring at the mean of the sample across measures, and who largely contributed to the group differences observed in pragmatic language and select social cognitive tasks and EF. Profile 2 included 17% of the sample, and was characterized by elevated and co-occurring mood and anxiety symptoms, mild expression of personality features of the BAP, and higher suprasegmental violations (e.g., atypical variation in intonation, volume, or rate of speech). This specific co-occurrence of features is not surprising in the context of social-emotional patterns commonly observed among individuals who are more anxious or depressed, in that their symptoms can interfere with their social relationships, and vice versa (120). Allen et al. (55) also reported distinct clusters of PM carriers who reported different mood and anxiety symptoms, and other work has reported the co-occurrence of mood/anxiety features with executive dysfunction among PM carriers (54). Consistent with prior work (121), this subgroup also exhibited slightly elevated BAP traits, which raises the possibility that the co-occurrence of such traits may also be common to a subgroup of family members of individuals with ASD (of note, individuals showing Profile 3, discussed below, demonstrated elevated BAP features in the absence of elevated mood/anxiety symptoms). Although suprasegmental speech violations committed by PM carriers in this subgroup does not constitute clinical impairment, among individuals with ASD, where suprasegmental atypicalities are more pronounced, such variation can pose a significant obstacle to social interactions (122). First-degree relatives of individuals with ASD also demonstrate subtle differences in suprasegmental aspects of language (e.g., prosody) (123, 124).

Profile 3 represented the smallest subgroup, comprised of ∼5% of PM participants who exhibited starker differences across all clinical-behavioral, social-cognitive, and executive domains relative to other PM carriers. This subgroup displayed notable differences on social-cognitive tasks together with elevated BAP features, and high rates of pragmatic language violations in particular. This pattern of performance is markedly similar to features described in prior investigations of parents of individuals with ASD who display the BAP (60, 61). Given the large number of ASD risk genes known to interact with *FMR1* (50, 51), it may be that this phenotypic profile reflects an increased genetic liability for ASD among this subgroup. However, the small size of Profile 3 warrants cautious interpretation. We found no group differences in age, IQ, or *FMR1*-related variation between the Profile subgroups, likely due in part to some missing data in Profile 3 and unequal sample sizes across groups, and further investigations in larger samples will be important for confirming these patterns. Importantly, membership within any of the profile groups was not associated with differences in age, IQ, or parenting stress-related factors (including number of affected children, or severity of child symptoms), suggesting the phenotypic profiles identified are likely reflective of inherent traits, rather than systematic environmental differences between subgroups.

Finally, some associations were detected between phenotypic profiles and *FMR1*-related molecular genetic variability in the PM carrier group. We found linear associations between CGG repeat length and pragmatic language, indicating greater violations at *lower* ends of the CGG continuum. The CGG range reflected in the participants studied extended from 59 to 126 CGGs; thus, it may be that inclusion of more PM carriers with higher repeats (i.e., over 120) might have altered the findings observed here. Prior work has observed curvilinear links between language and CGG length, with different patterns noted in the mid-range (90–110 repeats) as compared to those with CGG repeats beyond 120 (21). Interestingly, the linear CGG association became marginal after factoring in participants' activation ratios, suggesting the importance of considering the second, healthy X allele in phenotype-genotype associations of females with the *FMR1* PM. Additionally, we found that higher levels of FMRP predicted poorer performance on one social-cognitive task. This finding is somewhat in contrast to those from Hessl et al. (37), who found associations between *reduced* FMRP and poorer performance on social processing tasks in male PM carriers. Interestingly, a prior study based on the PM sample of participants studied here reported increased FMRP related to poorer performance on a language fluency task that taps into executive functioning (22), which together may suggest that the findings observed here may be specific to our sample of PM carriers (and thus may not be replicable), or that FMRP from blood is not analogous to FMRP in the brain. The links between *FMR1* and the phenotypes included in the present study are likely not straightforward, and it is possible that other *FMR1*-related factors (e.g., mosaicism, mRNA) could also help to elucidate *FMR1*-associated patterns not explored in this study (125, 126).

Strengths, Limitations, and Future Directions

Together, findings contribute to an emerging profile of PM carriers that suggests substantial phenotypic variability, and the presence of distinct phenotypic subgroups that may reveal important differences in underlying mechanistic factors and etiology (e.g., involvement of ASD risk genes interacting with *FMR1*). Strengths of this study included the broad phenotypic characterization of a relatively large group of females with the PM. Examining an array of phenotypic measures in a single sample enabled us to investigate comparisons with controls as well as unique profiles among PM carriers. Nevertheless, a larger sample may have mitigated some of the concerns with the small subgroup sample sizes yielded in our latent profile analyses.

We were limited in our attempts at a validation analysis of the three-profile model through comparison across such important factors as *FMR1*-related variation, though it is possible that other individual factors not available in the present study might have differentiated subgroups, such as direct measures of caregiving stress (127, 128), the presence of other co-occurring health conditions (55), or polygenic risk for ASD (47). There is emerging literature to suggest subgroups among PM carriers, and indeed the clinical disorders associated with the PM (i.e., FXTAS, FXPOI, and FXAND) only occur among a subset of PM carriers [e.g., see (55), and for review, (113, 129)]. Large scale studies of the PM are warranted to further investigate the interrelationships observed here, and to determine whether the phenotypes included in this study may co-occur with other meaningful clinical or health outcomes, as has been documented previously (55). Additionally, we were limited in *FMR1*-related information, as we did not have genetic data on the control group in this study. Further, there is emerging evidence to suggest that phenotypic associations may be observed across the range of CGG repeats (21, 130, 131). It may be that *FMR1* relationships with phenotypes were not detected given that the range of repeats in the current study was limited to the PM range. It may also be that molecular parameters in blood do not correlate with cognitive assays in the brain in straightforward ways. We recognize that due to our exclusion criteria, our control group might be expected to have performed well on the measures employed here. Larger and more heterogeneous control groups should be included in future studies, or in comparison to mothers of children with ASD. Finally, it will be important for future work to examine whether the findings reported here may extend to males with the PM, given prior evidence to suggest females and males with the PM may exhibit somewhat different phenotypic profiles and associations with underlying biology (29, 132). Such studies may build on the present findings, and help to characterize the phenotypic profile associated with the *FMR1* PM, and inform clinical efforts to promote the health and well-being of individuals with the PM and their families.

CONCLUSIONS

This study provided a comprehensive assessment of clinical and subclinical phenotypes associated with the *FMR1* PM. We identified differences from controls on pragmatic language features of the BAP, executive functioning, and some aspects of social cognition, but did not observe differences in mood and anxiety. Using LPA, we found subgroups within the PM sample characterized by unique patterns of performance on these measures. This study adds to a growing literature suggestive of important phenotypic heterogeneity among PM carriers, and provides further insight into *FMR1*-associated phenotypes.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: https://nda.nih.gov/edit_collection.html?id=1958.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Northwestern University Institutional Review Board. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

NM helped to conceptualize the project, drafted the initial manuscript, and analyzed the data. JG, KN, MW, and JL assisted in preparing the dataset and provided valuable feedback on manuscript drafts. GM contributed to manuscript preparation. LB, SP, and MLe contributed to data preparation and processing. JS provided further consultation and verification of statistical methods and results. DH advised on PM participant evaluation of neurocognitive functioning. LZ, KS, and EB-K provided resources for genetic analysis of the FMR1 gene, helped to draft the Materials and Methods section pertaining to genetic analyses, and helped with interpretation of results. MLo secured funding for the projects from which these data were drawn, conceptualized the project, helped to interpret data, and develop the manuscript. All authors reviewed and approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.718485/full#supplementary-material

REFERENCES

1. Nolin SL, Lewis FA, Ye LL, Houck GE, Glicksman AE, Limprasert P, et al. Familial transmission of the FMR1 CGG repeat. *Am J Hum Genet.* (1996) 59:1252–61.
2. Hagerman RJ, Hagerman PJ. The fragile X premutation: into the phenotypic fold. *Curr Opin Genet Dev.* (2002) 12:278–83. doi: 10.1016/S0959-437X(02)00299-X
3. Huber KM, Gallagher SM, Warren ST, Bear MF. Altered synaptic plasticity in a mouse model of fragile X mental retardation. *Proc Natl Acad Sci U S A.* (2002) 99:7746–50. doi: 10.1073/pnas.122205699
4. Sullivan AK, Marcus M, Epstein MP, Allen EG, Anido AE, Paquin JJ, et al. Association of FMR1 repeat size with ovarian dysfunction. *Hum Reprod.* (2005) 20:402–12. doi: 10.1093/humrep/deh635
5. Coffee B, Keith K, Albizua I, Malone T, Mowrey J, Sherman SL, et al. Incidence of fragile X syndrome by newborn screening for methylated FMR1 DNA. *Am J Hum Genet.* (2009) 85:503–14. doi: 10.1016/j.ajhg.2009.09.007
6. Hunter J, Rivero-Arias O, Angelov A, Kim E, Fotheringham I, Leal J. Epidemiology of fragile X syndrome: a systematic review and meta-analysis. *Am J Med Genet A.* (2014) 164:1648–58. doi: 10.1002/ajmg.a.36511
7. Wang LW, Berry-Kravis E, Hagerman RJ. Fragile X: leading the way for targeted treatments in autism. *Neurotherapeutics.* (2010) 7:264–74. doi: 10.1016/j.nurt.2010.05.005
8. Seltzer MM, Baker MW, Hong J, Maenner M, Greenberg J, Mandel D. Prevalence of CGG expansions of the FMR1 gene in a US population-based sample. *Am J Med Genet B Neuropsychiatr Genet.* (2012) 159:589–97. doi: 10.1002/ajmg.b.32065
9. Tassone F, Iong KP, Tong TH, Lo J, Gane LW, Berry-Kravis E, et al. FMR1 CGG allele size and prevalence ascertained through newborn screening in the United States. *Genome Med.* (2012) 4:100. doi: 10.1186/gm401
10. Maenner MJ, Baker MW, Broman KW, Tian J, Barnes JK, Atkins A, et al. FMR1 CGG expansions: prevalence and sex ratios. *Am J Med Genet B Neuropsychiatr Genet.* (2013) 162B:466–73. doi: 10.1002/ajmg.b.32176
11. Macpherson JN, Murray A. Development of genetic testing for fragile X syndrome and associated disorders, and estimates of the prevalence of FMR1 expansion mutations. *Genes (Basel).* (2016) 7:110. doi: 10.3390/genes7120110
12. Owens KM, Dohany L, Holland C, Dare J, Mann T, Settler C, et al. FMR1 premutation frequency in a large, ethnically diverse population referred for carrier testing. *Am J Med Genet A.* (2018) 176:1304–8. doi: 10.1002/ajmg.a.38692
13. Hagerman RJ, Hagerman P. Fragile X-associated tremor/ataxia syndrome - features, mechanisms and management. *Nat Rev Neurol.* (2016) 12:403–12. doi: 10.1038/nrneurol.2016.82
14. Hagerman RJ, Protic D, Rajaratnam A, Salcedo-Arellano MJ, Aydin EY, Schneider A. Fragile X-associated neuropsychiatric disorders (FXAND). *Front Psychiatry.* (2018) 9:564. doi: 10.3389/fpsyt.2018.00564
15. Tassone F, Hagerman RJ, Taylor AK, Gane LW, Godfrey TE, Hagerman PJ. Elevated levels of FMR1 mRNA in carrier males: a new mechanism of involvement in the fragile-X syndrome. *Am J Hum Genet.* (2000) 66:6–15. doi: 10.1086/302720
16. Kenneson A, Zhang F, Hagedorn CH, Warren ST. Reduced FMRP and increased FMR1 transcription is proportionally associated with CGG repeat number in intermediate-length and premutation carriers. *Hum Mol Genet.* (2001) 10:1449–54. doi: 10.1093/hmg/10.14.1449
17. Ennis S, Ward D, Murray A. Nonlinear association between CGG repeat number and age of menopause in FMR1 premutation carriers. *Eur J Hum Genet.* (2006) 14:253–5. doi: 10.1038/sj.ejhg.5201510
18. Seltzer MM, Abbeduto L, Greenberg JS, Almeida D, Hong J, Witt W. Biomarkers in the study of families of individuals with developmental disabilities. *Int Rev Res Ment Retard.* (2009) 37:213–49. doi: 10.1016/S0074-7750(09)37007-X
19. Hagerman R, Hagerman P. Advances in clinical and molecular understanding of the FMR1 premutation and fragile X-associated tremor/ataxia syndrome. *Lancet Neurol.* (2013) 12:786–98. doi: 10.1016/S1474-4422(13)70125-X
20. Mailick MR, Hong J, Greenberg J, Smith L, Sherman S. Curvilinear association of CGG repeats and age at menopause in women with FMR1 premutation expansions. *Am J Med Genet B Neuropsychiatr Genet.* (2014) 165B:705–11. doi: 10.1002/ajmg.b.32277
21. Klusek J, Porter A, Abbeduto L, Adayev T, Tassone F, Mailick MR, et al. Curvilinear association between language disfluency and FMR1 CGG repeat size across the normal, intermediate, and premutation range. *Front Genet.* (2018) 9:344. doi: 10.3389/fgene.2018.00344
22. Nayar K, Mckinney W, Hogan AL, Martin GE, La Valle C, Sharp K, et al. Language processing skills linked to FMR1 variation: a study of gaze-language coordination during rapid automatized naming among women with the FMR1 premutation. *PLoS One.* (2019) 14:e0219924. doi: 10.1371/journal.pone.0219924
23. Klusek J, Hong J, Sterling A, Berry-Kravis E, Mailick MR. Inhibition deficits are modulated by age and CGG repeat length in carriers of the FMR1 premutation allele who are mothers of children with fragile X syndrome. *Brain Cogn.* (2020) 139:105511. doi: 10.1016/j.bandc.2019.105511
24. Clifford S, Dissanayake C, Bui QM, Huggins R, Taylor AK, Loesch DZ. Autism spectrum phenotype in males and females with fragile X full mutation and premutation. *J Autism Dev Disord.* (2007) 37:738–47. doi: 10.1007/s10803-006-0205-z
25. Bailey DB, Raspa M, Olmsted M, Holiday DB. Co-occurring conditions associated with FMR1 gene variations: findings from a national parent survey. *Am J Med Genet A.* (2008) 146A:2060–9. doi: 10.1002/ajmg.a.32439
26. Bourgeois JA, Coffey SM, Rivera SM, Hessl D, Gane LW, Tassone F, et al. A review of fragile X premutation disorders: expanding the psychiatric perspective. *J Clin Psychiatry.* (2009) 70:852–62. doi: 10.4088/JCP.08r04476
27. Losh M, Klusek J, Martin GE, Sideris J, Parlier M, Piven J. Defining genetically meaningful language and personality traits in relatives of individuals with fragile X syndrome and relatives of individuals with autism. *Am J Med Genet B Neuropsychiatr Genet.* (2012) 159B:660–8. doi: 10.1002/ajmg.b.32070
28. Franke P, Leboyer M, Gänsicke M, Weiffenbach O, Biancalana V, Cornillet-Lefebre P, et al. Genotype-phenotype relationship in female carriers of the premutation and full mutation of FMR-1. *Psychiatry Res.* (1998) 80:113–27. doi: 10.1016/S0165-1781(98)00055-9
29. Hunter JE, Allen EG, Abramowitz A, Rusin M, Leslie M, Novak G, et al. Investigation of phenotypes associated with mood and anxiety among male and female fragile X premutation carriers. *Behav Genet.* (2008) 38:493–502. doi: 10.1007/s10519-008-9214-3
30. Roberts JE, Bailey DB, Mankowski J, Ford A, Sideris J, Weisenfeld LA, et al. Mood and anxiety disorders in females with the FMR1 premutation. *Am J Med Genet B Neuropsychiatr Genet.* (2009) 150B:130–9. doi: 10.1002/ajmg.b.30786
31. Bourgeois JA, Seritan AL, Casillas EM, Hessl D, Schneider A, Yang Y, et al. Lifetime prevalence of mood and anxiety disorders in fragile X premutation carriers. *J Clin Psychiatry.* (2011) 72:175–82. doi: 10.4088/JCP.09m05407blu
32. Cordeiro L, Abucayan F, Hagerman R, Tassone F, Hessl D. Anxiety disorders in fragile X premutation carriers: preliminary characterization of probands and non-probands. *Intractable Rare Dis Res.* (2015) 4:123–30. doi: 10.5582/irdr.2015.01029
33. Roberts JE, Tonnsen BL, Mccary LM, Ford AL, Golden RN, Bailey DB. Trajectory and predictors of depression and anxiety disorders in mothers with the FMR1 premutation. *Biol Psychiatry.* (2016) 79:850–7. doi: 10.1016/j.biopsych.2015.07.015
34. Cornish K, Kogan C, Turk J, Manly T, James N, Mills A, et al. The emerging fragile X premutation phenotype: evidence from the domain of social cognition. *Brain Cogn.* (2005) 57:53–60. doi: 10.1016/j.bandc.2004.08.020
35. Hessl D, Rivera S, Koldewyn K, Cordeiro L, Adams J, Tassone F, et al. Amygdala dysfunction in men with the fragile X premutation. *Brain.* (2007) 130:404–16. doi: 10.1093/brain/awl338
36. Hunter JE, Abramowitz A, Rusin M, Sherman SL. Is there evidence for neuropsychological and neurobehavioral phenotypes among adults without FXTAS who carry the FMR1 premutation? A review of current literature. *Genet Med.* (2009) 11:79–89. doi: 10.1097/GIM.0b013e31818de6ee
37. Hessl D, Wang JM, Schneider A, Koldewyn K, Le L, Iwahashi C, et al. Decreased fragile X mental retardation protein expression underlies amygdala dysfunction in carriers of the fragile X premutation. *Biol Psychiatry.* (2011) 70:859–65. doi: 10.1016/j.biopsych.2011.05.033

38. Shelton AL, Cornish KM, Kolbe S, Clough M, Slater HR, Li X, et al. Brain structure and intragenic DNA methylation are correlated, and predict executive dysfunction in fragile X premutation females. *Transl Psychiatry.* (2016) 6:e984. doi: 10.1038/tp.2016.250
39. Abbeduto L, Mcduffie A, Thurman AJ. The fragile X syndrome-autism comorbidity: what do we really know? *Front Genet.* (2014) 5:355. doi: 10.3389/fgene.2014.00355
40. Yu TW, Berry-Kravis E. Autism and fragile X syndrome. *Semin Neurol.* (2014) 34:258–65. doi: 10.1055/s-0034-1386764
41. Thurman AJ, Mcduffie A, Kover ST, Hagerman RJ, Abbeduto L. Autism symptomatology in boys with fragile X syndrome: a cross sectional developmental trajectories comparison with nonsyndromic autism spectrum disorder. *J Autism Dev Disord.* (2015) 45:2816–32. doi: 10.1007/s10803-015-2443-4
42. Kaufmann WE, Kidd SA, Andrews HF, Budimirovic DB, Esler A, Haas-Givler B, et al. Autism spectrum disorder in fragile X syndrome: cooccurring conditions and current treatment. *Pediatrics.* (2017) 139:S194–S206. doi: 10.1542/peds.2016-1159F
43. Farzin F, Perry H, Hessl D, Loesch D, Cohen J, Bacalman S, et al. Autism spectrum disorders and attention-deficit/hyperactivity disorder in boys with the fragile X premutation. *J Dev Behav Pediatr.* (2006) 27:S137–S144. doi: 10.1097/00004703-200604002-00012
44. Piven J, Palmer P, Landa R, Santangelo SJD, Childress D. Personality and language characteristics in parents from multiple-incidence autism families. *Am J Med Genet Pt B.* (1997) 74:398–411. doi: 10.1002/(SICI)1096-8628(19970725)74:4<398::AID-AJMG11>3.0.CO;2-D
45. Murphy M, Bolton P, Pickles A, Fombonne E, Piven J, Rutter M. Personality traits of the relatives of autistic probands. *Psychol Med.* (2000) 30:1411–24. doi: 10.1017/S0033291799002949
46. Losh M, Childress D, Lam K, Piven J. Defining key features of the broad autism phenotype: a comparison across parents of multiple- and single-incidence autism families. *Am J Med Genet B Neuropsychiatr Genet.* (2008) 147B:424–33. doi: 10.1002/ajmg.b.30612
47. Nayar K, Sealock JM, Maltman N, Bush L, Cook EH, Davis LK, et al. Elevated polygenic burden for autism spectrum disorder is associated with the broad autism phenotype in mothers of individuals with autism spectrum disorder. *Biol Psychiatry.* (2021) 89:476–85. doi: 10.1016/j.biopsych.2020.08.029
48. Tyrer P (editor). Personality assessment schedule. In: *Personality Disorders: Diagnosis, Management, and Course.* London: Butterworth and Company (1988). p. 43–62.
49. Landa R, Piven J, Wzorek MM, Gayle JO, Chase GA, Folstein SE. Social language use in parents of autistic individuals. *Psychol Med.* (1992) 22:245–54. doi: 10.1017/S0033291700032918
50. Darnell JC, Van Driesche SJ, Zhang C, Hung KY, Mele A, Fraser CE, et al. FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell.* (2011) 146:247–61. doi: 10.1016/j.cell.2011.06.013
51. Iossifov I, Ronemus M, Levy D, Wang Z, Hakker I, Rosenbaum J, et al. *De novo* gene disruptions in children on the autistic spectrum. *Neuron.* (2012) 74:285–99. doi: 10.1016/j.neuron.2012.04.009
52. Gossett A, Sansone S, Schneider A, Johnston C, Hagerman R, Tassone F, et al. Psychiatric disorders among women with the fragile X premutation without children affected by fragile X syndrome. *Am J Med Genet B Neuropsychiatr Genet.* (2016) 171:1139–47. doi: 10.1002/ajmg.b.32496
53. Jiraanont P, Sweha SR, Alolaby RR, Silva M, Tang HT, Durbin-Johnson B, et al. Clinical and molecular correlates in fragile X premutation females. *eNeurologicalSci.* (2017) 7:49–56. doi: 10.1016/j.ensci.2017.04.003
54. Kraan CM, Hocking DR, Georgiou-Karistianis N, Metcalfe SA, Archibald AD, Fielding J, et al. Impaired response inhibition is associated with self-reported symptoms of depression, anxiety, and ADHD in female FMR1 premutation carriers. *Am J Med Genet B Neuropsychiatr Genet.* (2014) 165B:41–51. doi: 10.1002/ajmg.b.32203
55. Allen EG, Charen K, Hipp HS, Shubeck L, Amin A, He W, et al. Clustering of comorbid conditions among women who carry an FMR1 premutation. *Genet Med.* (2020) 22:758–66. doi: 10.1038/s41436-019-0733-5
56. Schneider A, Johnston C, Tassone F, Sansone S, Hagerman RJ, Ferrer E, et al. Broad autism spectrum and obsessive-compulsive symptoms in adults with the fragile X premutation. *Clin Neuropsychol.* (2016) 30:929–43. doi: 10.1080/13854046.2016.1189536
57. Klusek J, Schmidt J, Fairchild AJ, Porter A, Roberts JE. Altered sensitivity to social gaze in the FMR1 premutation and pragmatic language competence. *J Neurodev Disord.* (2017) 9:31. doi: 10.1186/s11689-017-9211-z
58. Capps L, Kehres J, Sigman M. Conversational abilities among children with autism and children with developmental delays. *Autism.* (1998) 2:325–44. doi: 10.1177/1362361398024002
59. Losh M, Capps L. Narrative ability in high-functioning children with autism or Asperger's syndrome. *J Autism Dev Disord.* (2003) 33:239–51. doi: 10.1023/A:1024446215446
60. Losh M, Piven J. Social-cognition and the broad autism phenotype: identifying genetically meaningful phenotypes. *J Child Psychol Psychiatry.* (2007) 48:105–12. doi: 10.1111/j.1469-7610.2006.01594.x
61. Losh M, Adolphs R, Poe MD, Couture S, Penn D, Baranek GT, et al. Neuropsychological profile of autism and the broad autism phenotype. *Arch Gen Psychiatry.* (2009) 66:518–26. doi: 10.1001/archgenpsychiatry.2009.34
62. Losh M, Martin GE, Klusek J, Hogan-Brown AL, Sideris J. Social communication and theory of mind in boys with autism and fragile x syndrome. *Front Psychol.* (2012) 3:266. doi: 10.3389/fpsyg.2012.00266
63. Winston M, Nayar K, Hogan AL, Barstein J, La Valle C, Sharp K, et al. Physiological regulation and social-emotional processing in female carriers of the FMR1 premutation. *Physiol Behav.* (2020) 214:112746. doi: 10.1016/j.physbeh.2019.112746
64. Winston M, Nayar K, Landau E, Maltman N, Sideris J, Zhou L, et al. A unique visual attention profile associated with the FMR1 premutation. *Front Genet.* (2021) 12:591211. doi: 10.3389/fgene.2021.591211
65. Grigsby J, Brega AG, Engle K, Leehey MA, Hagerman RJ, Tassone F, et al. Cognitive profile of fragile X premutation carriers with and without fragile X-associated tremor/ataxia syndrome. *Neuropsychology.* (2008) 22:48–60. doi: 10.1037/0894-4105.22.1.48
66. Grigsby J, Cornish K, Hocking D, Kraan C, Olichney JM, Rivera SM, et al. The cognitive neuropsychological phenotype of carriers of the FMR1 premutation. *J Neurodev Disord.* (2014) 6:28. doi: 10.1186/1866-1955-6-28
67. Baker EK, Arpone M, Aliaga SM, Bretherton L, Kraan CM, Bui M, et al. Incomplete silencing of full mutation alleles in males with fragile X syndrome is associated with autistic features. *Mol Autism.* (2019) 10:21. doi: 10.1186/s13229-019-0271-7
68. Shelton AL, Cornish KM, Godler DE, Clough M, Kraan C, Bui M, et al. Delineation of the working memory profile in female FMR1 premutation carriers: the effect of cognitive load on ocular motor responses. *Behav Brain Res.* (2015) 282:194–200. doi: 10.1016/j.bbr.2015.01.011
69. Shelton AL, Cornish K, Fielding J. Long term verbal memory recall deficits in fragile X premutation females. *Neurobiol Learn Mem.* (2017) 144:131–5. doi: 10.1016/j.nlm.2017.07.002
70. Shelton AL, Cornish K, Kraan C, Georgiou-Karistianis N, Metcalfe SA, Bradshaw JL, et al. Exploring inhibitory deficits in female premutation carriers of fragile X syndrome: through eye movements. *Brain Cogn.* (2014) 85:201–8. doi: 10.1016/j.bandc.2013.12.006
71. Goodrich-Hunsaker NJ, Wong LM, Mclennan Y, Tassone F, Harvey D, Rivera SM, et al. Adult female Fragile X Premutation carriers exhibit age- and CGG repeat length-related impairments on an attentionally based enumeration task. *Front Hum Neurosci.* (2011) 5:63. doi: 10.3389/fnhum.2011.00063
72. Jacquemont S, Hagerman RJ, Leehey M, Grigsby J, Zhang L, Brunberg JA, et al. Fragile X premutation tremor/ataxia syndrome: molecular, clinical, and neuroimaging correlates. *Am J Hum Genet.* (2003) 72:869–78. doi: 10.1086/374321
73. Moore CJ, Daly EM, Schmitz N, Tassone F, Tysoe C, Hagerman RJ, et al. A neuropsychological investigation of male premutation carriers of fragile X syndrome. *Neuropsychologia.* (2004) 42:1934–47. doi: 10.1016/j.neuropsychologia.2004.05.002
74. Sterling A, Mailick M, Greenberg J, Warren SF, Brady N. Language dysfluencies in females with the FMR1 premutation. *Brain Cogn.* (2013) 82:84–9. doi: 10.1016/j.bandc.2013.02.009
75. Johnston C, Eliez S, Dyer-Friedman J, Hessl D, Glaser B, Blasey C, et al. Neurobehavioral phenotype in carriers of the fragile X premutation. *Am J Med Genet.* (2001) 103:314–9. doi: 10.1002/ajmg.1561

76. Goodlin-Jones BL, Tassone F, Gane LW, Hagerman RJ. Autistic spectrum disorder and the fragile X premutation. *J Dev Behav Pediatr.* (2004) 25:392–8. doi: 10.1097/00004703-200412000-00002
77. Loesch DZ, Bui MQ, Hammersley E, Schneider A, Storey E, Stimpson P, et al. Psychological status in female carriers of premutation FMR1 allele showing a complex relationship with the size of CGG expansion. *Clin Genet.* (2015) 87:173–8. doi: 10.1111/cge.12347
78. Leehey MA, Berry-Kravis E, Goetz CG, Zhang L, Hall DA, Li L, et al. FMR1 CGG repeat length predicts motor dysfunction in premutation carriers. *Neurology.* (2008) 70:1397–402. doi: 10.1212/01.wnl.0000281692.98200.f5
79. Hall DA, Stebbins GT, Jacquemont S, Berry-Kravis E, Goetz CG, Hagerman R, et al. Clinimetric properties of the fragile X-associated Tremor Ataxia Syndrome Rating Scale. *Mov Disord Clin Pract.* (2019) 6:120–4. doi: 10.1002/mdc3.12708
80. Sasson NJ, Lam KS, Childress D, Parlier M, Daniels JL, Piven J. The broad autism phenotype questionnaire: prevalence and diagnostic classification. *Autism Res.* (2013) 6:134–43. doi: 10.1002/aur.1272
81. Beck AT, Steer RA, Brown GK. *Manual for the Beck Depression Inventory-II*. San Antonio, TX: Psychological Corporation (1996).
82. Spielberger CD, Gorsuch RL, Lushene R, Vagg PR, Jacobs GA. *Manual for the State-Trait Anxiety Inventory*. Palo Alto, CA: Consulting Psychologists Press (1983).
83. Knight RG, Waal-Manning HJ, Spears GF. Some norms and reliability data for the State–Trait Anxiety Inventory and the Zung Self-Rating Depression scale. *Br J Clin Psychol.* (1983) 22 (Pt 4):245–9. doi: 10.1111/j.2044-8260.1983.tb00610.x
84. Julian LJ. Measures of anxiety: State-Trait Anxiety Inventory (STAI), Beck Anxiety Inventory (BAI), and Hospital Anxiety and Depression Scale-Anxiety (HADS-A). *Arthritis Care Res (Hoboken).* (2011) 63(Suppl. 11):S467–S72. doi: 10.1002/acr.20561
85. Sheehan DV, Lecrubier Y, Sheehan KH, Amorim P, Janavs J, Weiller E, et al. The Mini-International Neuropsychiatric Interview (M.I.N.I.): the development and validation of a structured diagnostic psychiatric interview for DSM-IV and ICD-10. *J Clin Psychiatry.* (1998) 59(Suppl. 20):22–33; quiz 34–57.
86. Baron-Cohen S, Wheelwright S, Hill J, Raste Y, Plumb I. The "reading the mind in the eyes" test revised version: a study with normal adults, and adults with Asperger syndrome or high-functioning autism. *J Child Psychol Psychiatry Allied Discipl.* (2001) 42:241–51. doi: 10.1111/1469-7610.00715
87. Adolphs R, Tranel D, Damasio AR. The human amygdala in social judgment. *Nature.* (1998) 393:470–4. doi: 10.1038/30982
88. Adolphs R, Sears L, Piven J. Abnormal processing of social information from faces in autism. *J Cogn Neurosci.* (2001) 13:232–40. doi: 10.1162/089892901564289
89. Adolphs R, Tranel D. Amygdala damage impairs emotion recognition from scenes only when they contain facial expressions *Neuropsychologia.* (2003) 41:1281–9. doi: 10.1016/S0028-3932(03)00064-2
90. Heberlein A, Ravahi S, Adolphs R, Tranel D. Deficits in attributing emotion to moving visual stimuli consequent to amygdala damage. *Cogn Neurosci Soc Abstracts.* (2000) 2000:56.
91. Roth RM, Isquith PK, Gioia GA. *Behavior Rating Inventory of Executive Function-Adult Version*. Lutz, FL: Psychological Assessment Resources (2005).
92. Lafauci G, Adayev T, Kascsak R, Nolin S, Mehta P, Brown WT, et al. Fragile X screening by quantification of FMRP in dried blood spots by a Luminex immunoassay. *J Mol Diagn.* (2013) 15:508–17. doi: 10.1016/j.jmoldx.2013.02.006
93. Berry-Kravis E, Lewin F, Wuu J, Leehey M, Hagerman R, Hagerman P, et al. Tremor and ataxia in fragile X premutation carriers: blinded videotape study. *Ann Neurol.* (2003) 53:616–23. doi: 10.1002/ana.10522
94. Cohen J. *Statistical Power Analysis for the Behavioral Sciences*. New York, NY: Routledge Academic (1988).
95. Spurk D, Hirschi A, Wang M, Valero D, Kauffeld S. Latent profile analysis: a review and "how to" guide of its application within vocational behavior research. *J Vocat Behav.* (2020) 120:103445. doi: 10.1016/j.jvb.2020.103445
96. Tein JY, Coxe S, Cham H. Statistical power to detect the correct number of classes in latent profile analysis. *Struct Equ Model.* (2013) 20:640–57. doi: 10.1080/10705511.2013.824781
97. Williams GA, Kibowski F. Latent class analysis and latent profile analysis. In: Jason LA, Glenwick DS, editors. *Handbook of Methodological Approaches to Community-Based Research: Qualitative, Quantitative, and Mixed Methods*. New York, NY: Oxford University Press (2016). p. 143–151.
98. Ferguson SL, Whitney E, Moore G, Hull D. Finding latent groups in observed data: a primer on latent profile analysis in Mplus for applied researchers. *Int J Behav Dev.* (2019) 44:458–68. doi: 10.1177/0165025419881721
99. Lord C, Rutter M, Dilavore PC, Risi S, Gotham K, Bishop S. *Autism Diagnostic Observation Schedule, 2nd Edn*. Torrance, CA: Western Psychological Services (2012).
100. Klusek J, Mcgrath SE, Abbeduto L, Roberts JE. Pragmatic language features of mothers with the FMR1 premutation are associated with the language outcomes of adolescents and young adults with fragile X syndrome. *J Speech Lang Hear Res.* (2016) 59:49–61. doi: 10.1044/2015_JSLHR-L-15-0102
101. Klusek J, Fairchild AJ, Roberts JE. Vagal tone as a putative mechanism for pragmatic competence: an investigation of carriers of the FMR1 premutation. *J Autism Dev Disord.* (2019) 49:197–208. doi: 10.1007/s10803-018-3714-7
102. Klusek J, Thurman AJ, Abbeduto L. Maternal pragmatic language difficulties in the FMR1 premutation and the broad autism phenotype: associations with individual and family outcomes. *J Autism Dev Disord.* (2021) 59:49–61. doi: 10.1007/s10803-021-04980-3
103. Abbeduto L, Brady N, Kover ST. Language development and fragile X syndrome: profiles, syndrome-specificity, and within-syndrome differences. *Ment Retard Dev Disabil Res Rev.* (2007) 13:36–46. doi: 10.1002/mrdd.20142
104. Kover ST, Abbeduto L. Expressive language in male adolescents with fragile X syndrome with and without comorbid autism. *J Intellect Disabil Res.* (2010) 54:246–65. doi: 10.1111/j.1365-2788.2010.01255.x
105. Hogan-Brown AL, Losh M, Martin GE, Mueffelmann DJ. An investigation of narrative ability in boys with autism and fragile X syndrome. *Am J Intellect Dev Disabil.* (2013) 118:77–94. doi: 10.1352/1944-7558-118.2.77
106. Martin GE, Losh M, Estigarribia B, Sideris J, Roberts J. Longitudinal profiles of expressive vocabulary, syntax and pragmatic language in boys with fragile X syndrome or Down syndrome. *Int J Lang Commun Disord.* (2013) 48:432–43. doi: 10.1111/1460-6984.12019
107. Klusek J, Martin GE, Losh M. A comparison of pragmatic language in boys with autism and fragile X syndrome. *J Speech Lang Hear Res.* (2014) 57:1692–707. doi: 10.1044/2014_JSLHR-L-13-0064
108. Martin GE, Barstein J, Hornickel J, Matherly S, Durante G, Losh M. Signaling of noncomprehension in communication breakdowns in fragile X syndrome, down syndrome, and autism spectrum disorder. *J Commun Disord.* (2017) 65:22–34. doi: 10.1016/j.jcomdis.2017.01.003
109. Martin GE, Bush L, Klusek J, Patel S, Losh M. A multimethod analysis of pragmatic skills in children and adolescents with fragile X syndrome, autism spectrum disorder, and down syndrome. *J Speech Lang Hear Res.* (2018) 61:3023–37. doi: 10.1044/2018_JSLHR-L-18-0008
110. Kessler RC, Berglund P, Demler O, Jin R, Koretz D, Merikangas KR, et al. The epidemiology of major depressive disorder: results from the National Comorbidity Survey Replication (NCS-R). *JAMA.* (2003) 289:3095–105. doi: 10.1001/jama.289.23.3095
111. Lewis P, Abbeduto L, Murphy M, Richmond E, Giles N, Bruno L, et al. Cognitive, language and social-cognitive skills of individuals with fragile X syndrome with and without autism. *J Intellect Disabil Res.* (2006) 50:532–45. doi: 10.1111/j.1365-2788.2006.00803.x
112. Adolphs R. Social cognition and the human brain. *Trends Cogn Sci.* (1999) 3:469–79. doi: 10.1016/s1364-6613(99)01399-6
113. Wheeler AC, Bailey DB, Berry-Kravis E, Greenberg J, Losh M, Mailick M, et al. Associated features in females with an FMR1 premutation. *J Neurodev Disord.* (2014) 6:30. doi: 10.1186/1866-1955-6-30
114. Brega AG, Goodrich G, Bennett RE, Hessl D, Engle K, Leehey MA, et al. The primary cognitive deficit among males with fragile X-associated tremor/ataxia syndrome (FXTAS) is a dysexecutive syndrome. *J Clin Exp Neuropsychol.* (2008) 30:853–69. doi: 10.1080/13803390701819044
115. Shelton AL, Cornish KM, Kraan CM, Lozano R, Bui M, Fielding J. Executive dysfunction in female FMR1 premutation carriers. *Cerebellum.* (2016) 15:565–9. doi: 10.1007/s12311-016-0782-0

116. Shelton AL, Cornish K, Clough M, Gajamange S, Kolbe S, Fielding J. Disassociation between brain activation and executive function in fragile X premutation females. *Hum Brain Mapp.* (2017) 38:1056–67. doi: 10.1002/hbm.23438
117. Movaghar A, Mailick M, Sterling A, Greenberg J, Saha K. Automated screening for Fragile X premutation carriers based on linguistic and cognitive computational phenotypes. *Sci Rep.* (2017) 7:2674. doi: 10.1038/s41598-017-02682-4
118. Birch RC, Hocking DR, Trollor JN. Prevalence and predictors of subjective memory complaints in adult male carriers of the FMR1 premutation. *Clin Neuropsychol.* (2016) 30:834–48. doi: 10.1080/13854046.2016.1145905
119. Hall D, Todorova-Koteva K, Pandya S, Bernard B, Ouyang B, Walsh M, et al. Neurological and endocrine phenotypes of fragile X carrier women. *Clin Genet.* (2016) 89:60–7. doi: 10.1111/cge.12646
120. Steger MF, Kashdan TB. Depression and everyday social activity, belonging, and well-being. *J Couns Psychol.* (2009) 56:289–300. doi: 10.1037/a0015416
121. Wallace GL, Budgett J, Charlton RA. Aging and autism spectrum disorder: evidence from the broad autism phenotype. *Autism Res.* (2016) 9:1294–303. doi: 10.1002/aur.1620
122. Mesibov GB. Treatment issues with high-functioning adolescents and adults with autism. In: Schopler E, Mesibov GB, editors. *High Functioning Individuals With Autism. Current Issues in Autism.* Boston, MA: Springer (1992). p. 143–55.
123. Patel SP, Kim JH, Larson CR, Losh M. Mechanisms of voice control related to prosody in autism spectrum disorder and first-degree relatives. *Autism Res.* (2019) 12:1192–210. doi: 10.1002/aur.2156
124. Patel SP, Nayar K, Martin GE, Franich K, Crawford S, Diehl JJ, et al. An acoustic characterization of prosodic differences in autism spectrum disorder and first-degree relatives. *J Autism Dev Disord.* (2020) 50:3032–45. doi: 10.1007/s10803-020-04392-9
125. Klusek J, Lafauci G, Adayev T, Brown WT, Tassone F, Roberts JE. Reduced vagal tone in women with the FMR1 premutation is associated with FMR1 mRNA but not depression or anxiety. *J Neurodev Disord.* (2017) 9:16. doi: 10.1186/s11689-017-9197-6
126. Mailick MR, Movaghar A, Hong J, Greenberg JS, Dawalt LS, Zhou L, et al. Health profiles of mosaic versus non-mosaic FMR1 premutation carrier mothers of children with fragile X syndrome. *Front Genet.* (2018) 9:173. doi: 10.3389/fgene.2018.00173
127. Bailey DB, Sideris J, Roberts J, Hatton D. Child and genetic variables associated with maternal adaptation to fragile X syndrome: a multidimensional analysis. *Am J Med Genet A.* (2008) 146A:720–9. doi: 10.1002/ajmg.a.32240
128. Seltzer MM, Barker ET, Greenberg JS, Hong J, Coe C, Almeida D. Differential sensitivity to life stress in FMR1 premutation carrier mothers of children with fragile X syndrome. *Health Psychol.* (2012) 31:612–22. doi: 10.1037/a0026528
129. Wheeler A, Raspa M, Hagerman R, Mailick M, Riley C. Implications of the FMR1 premutation for children, adolescents, adults, and their families. *Pediatrics.* (2017) 139:S172–S82. doi: 10.1542/peds.2016-1159D
130. Mailick MR, Hong J, Rathouz P, Baker MW, Greenberg JS, Smith L, et al. Low-normal FMR1 CGG repeat length: phenotypic associations. *Front Genet.* (2014) 5:309. doi: 10.3389/fgene.2014.00309
131. Adamsheck HC, Petty EM, Hong J, Baker MW, Brilliant MH, Mailick MR. Is low FMR1 CGG repeat length in males correlated with family history of BRCA-associated cancers? An exploratory analysis of medical records. *J Genet Couns.* (2017) 26:1401–10. doi: 10.1007/s10897-017-0116-5
132. Allen EG, Hunter JE, Rusin M, Juncos J, Novak G, Hamilton D, et al. Neuropsychological findings from older premutation carrier males and their noncarrier siblings from families with fragile X syndrome. *Neuropsychology.* (2011) 25:404–11. doi: 10.1037/a0021879

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Is *FMR1* CGG Repeat Number Polymorphism Associated With Phenotypic Variation in the General Population? Report From a Cohort of 5,499 Adults

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FMR1 CGG repeat length was assayed in 5499 research participants (2637 men and 2862 women) in the Wisconsin Longitudinal Study (WLS), a population-based cohort. Most past research has focused on clinically-ascertained individuals with expansions in CGG repeats, either those with fragile X syndrome (> 200 CGG repeats), the *FMR1* premutation (55–200 repeats), or in the gray zone (variously defined as 45–54 or 41–54 repeats). In contrast, the WLS is a unique source of data that was obtained from an unselected cohort of individuals from the general population for whom *FMR1* CGG repeat length was assayed. The WLS is a random sample of one-third of all high school seniors in the state of Wisconsin in 1957. The most recent round of data collection was in 2011; thus, the study spanned over 50 years. Saliva samples were obtained from 69% of surviving members of the cohort in 2008 and 2011, from which CGG repeats were assayed. With one exception, the CGG repeat length of all members of this cohort was below 100 (ranging from 7 to 84). The present study evaluated the genotype-phenotype associations of CGG repeat number and IQ, college graduation, age at menopause, number of biological children, having a child with intellectual or developmental disabilities, and the likelihood of experiencing an episode of depression during adulthood. Linear and curvilinear effects were probed. Although effect sizes were small, significant associations were found between CGG repeat length and high school IQ score, college graduation, number of biological children, age at menopause, and the likelihood of having an episode of depression. However, there was no significant association between repeat length and having a child diagnosed with an IDD condition. This study demonstrates a continuum of phenotype effects with *FMR1* repeat lengths and illustrates how research inspired by a rare genetic condition (such as fragile X syndrome) can be used to probe genotype-phenotype associations in the general population.

Keywords: *FMR1* CGG repeats, genotype-phenotype associations, genetic epidemiology, population cohorts, normal genetic variation

INTRODUCTION

The *FMR1* gene on the X chromosome plays a critically important role in the development and functioning of the nervous system, as its protein product normally regulates the translation of ∼30% of all transcripts in the pre- and post-synaptic proteomes critical for synaptic plasticity (1). Expansions above a critical threshold of a CGG triplet repeat (i.e., 200 CGG repeats) in the 5′ untranslated region cause fragile X syndrome (FXS), the most common inherited form of intellectual disability and autism. Additionally, repeats in the premutation range (55–200 repeats) and to a lesser extent in the gray zone (variously defined as 45–54 or 41–54 repeats) have received research attention leading to observation of phenotypic associations (2, 3).

However, the number of CGG repeats in *FMR1* is highly polymorphic in the human population (4, 5). The peak value for CGG repeats is at 30, with >90% of individuals having fewer than 40 and the lowest being 6 repeats (4, 6–8). In 2003, Chen et al. (7) reported the results from studies of synthetic human *FMR1* promoter sequences driving a luciferase reporter in transfected cell lines, showing that CGG repeats had no significant effect on transcription. However, compared to the modal number of approximately 30 CGGs, both lower and higher numbers of CGG repeats negatively affected translation of luciferase mRNA into protein, suggesting that the modal number of 30 may maximize translational efficiency. This observation predicts that repeats across the CGG range, above and below 30, may be associated with varying degrees in the efficiency of translation of the *FMR1* transcript, and thus may be associated with phenotypic variability. Wang et al. (9) have suggested that even subtle changes in both protein and mRNA levels could have wide-ranging effects both on brain structure and working memory in healthy adult men with normal *FMR1* alleles.

Most studies of genotype-phenotype associations in *FMR1* are based on data derived from clinical populations. Following diagnosis of a child with FXS, the family may be tested to determine if each member has expansions in the number of CGG repeats or is in the normal range. Thus, the majority of variation in CGG repeat number is not evaluated in these studies because only those at the very high end of the repeat range, and their relatives, are generally assayed. Understanding the true significance of the variations across much of the yet-unstudied range of *FMR1* CGG repeats would be advanced by study of a population-based sample.

The Wisconsin Longitudinal Study (WLS) is a unique source of phenotypic data that has been genotyped for CGG repeats in *FMR1*. It is a random sample of a cohort (mostly born in 1939), initially studied as high school seniors in 1957 and subsequently studied periodically through age 71 (10). Capitalizing on the availability of DNA isolated from saliva samples collected in 2008 and 2011, the *FMR1* CGG repeat number of the members of this cohort was ascertained, enabling study of genotype-phenotype associations across much of the CGG repeat range in a population-based sample of adults.

METHOD

The Present Study

The key question asked in the present study was, to what extent is polymorphism in *FMR1* CGG repeat number (which in this analysis ranged from 7 to 84 repeats) associated with individual variation in phenotypic characteristics? Although there have been previous studies of genotype-phenotype associations using *FMR1* CGG repeat data derived from participants in the WLS, there are three major differences between the previously reported WLS studies and the present research.

First, all past studies of genotype-phenotype associations in WLS included both the original cohort of Wisconsin 1957 high school graduates (referred to as the "graduates") as well as a sub-set of their siblings. However, in the present study, we analyze data from the graduate cohort only, not including the sibling participants. Whereas the majority of the members of the original cohort of 1957 high school graduates were born in 1939, their siblings who participated in the WLS (including step- and half-siblings) spanned the birth years of 1906 to 1970. Because some of the key phenotypes analyzed for the present study may have reflected societal trends that change over time (e.g., college attendance, number of children), we focused on an age cohort (i.e., 1957 high school graduates) to control for the influence of these secular trends.

Second, all prior WLS reports that analyzed *FMR1* CGG repeat data included only those participants who provided saliva samples in 2008 (n = 4,382 graduates provided samples). In 2011, an additional 1,118 graduates who had not responded to the original request provided such samples. Together, the samples obtained in 2008 and 2011 (n = 5,500) constituted DNA from 69% of the surviving members of the original cohort of high school graduates, all of which were assayed for *FMR1* CGG repeats. The present study's analysis of genotype-phenotype associations is the first to include data from all participants assayed for *FMR1* CGG repeats. However, for reasons described below, one case was dropped from the present analysis, resulting in an analytic sample of 5,499 adults.

Lastly, the statistical approach used in our past studies contrasted specific clinically-defined segments of the CGG repeat distribution (e.g., premutation vs. controls) (11), contrasted statistically-defined segments of the repeat distribution (e.g., "low zone" vs. controls) (12), or evaluated interaction effects between repeat number and environmental factors (e.g., parenting a child with a disability) (13). In contrast, the present analysis treated CGG repeats as a continuous variable and directly probed genotype-phenotype associations. Thus, the central question of the present study was whether *FMR1* CGG repeat number is associated with phenotypic characteristics in the general population, not in the context of stress exposure or in clinically- or statistically-defined segments of the repeat distribution.

Hypotheses

The hypotheses advanced in this study were based on past research examining three specific phenotypes that have been implicated in past research on *FMR1* CGG repeats below the full

mutation—cognitive, reproductive, and psychiatric phenotypes. However, most past research has focused on the upper end of the repeat range (i.e., gray zone and premutation expansions), with only a small number of studies extending across the full CGG repeat range (13–16) or specifically probing the low end of the repeat distribution (12). Although we predicted directional associations between *FMR1* CGG repeat number and specific phenotypic characteristics, the study was exploratory as, to the best of our knowledge, no past research has been conducted on a large unselected population-based cohort without clinical ascertainment of cases. Small effect sizes were expected.

Regarding the cognitive phenotype, we hypothesized that there would be a negative association between CGG repeat number and *IQ score*, based on past research showing that men and women who had greater numbers of CGG repeats have subtle limitations in cognitive functioning, including executive functioning limitations (3, 17, 18). We further expected a similar association for achieving a *college degree*, although this hypothesis was tested for men only, for reasons explained below.

Regarding the reproductive phenotype, the primary measure of women's fertility was *age at menopause*. Additionally, we evaluated the *number of biological children* born to women. We predicted that there would be negative associations between CGG repeat number and age at menopause, and also with number of biological children, based on past research that reported early menopause and infertility in women with CGG expansions in the premutation range (19–23). We also explored whether women at the higher end of the CGG distribution would be more likely to have *a child with a developmental disability* (24).

Regarding the psychiatric phenotype, past research reported higher rates of *depression* in men and women who had a higher number of CGG repeats (3, 21, 25). Therefore, we expected a positive association between CGG repeats and experiencing an episode of depression.

Much past research on genotype-phenotype associations in *FMR1* reported curvilinear associations, particularly for fertility-related and psychiatric phenotypes (13, 15, 19, 26, 27). In contrast, phenotypes associated with FXTAS (Fragile X-associated Tremor /Ataxia Syndrome) have shown a linear association between repeat number and symptoms (28, 29). Therefore, both linear and curvilinear associations were probed in this study.

Study Population and Data

Data for the present study were drawn from the WLS, a public use data set. It consists of a random sample of 10,317 women and men who graduated from Wisconsin high schools in 1957, representing one-third of that cohort (10). In 1957, 75% of Wisconsin residents who were of high school age graduated from high school. Follow-up studies were conducted in 1975 with 9138 members of the original cohort when they were, on average, 36-years old; in 1992 with 8493 respondents when they were in their early 50s; in 2004 with 7265 respondents when they were in their mid-60s; and again in 2011 with 5967 respondents when they were in their early 70s. The participants in the 2011 study constituted 72.2% of the surviving members of the original cohort.

Although all of the original WLS participants were high school graduates, these WLS participants ranged in IQ score from a low of 61 to a high of 145. Fully 15% had IQ scores of 85 (one SD below the mean) or below. This percentage is nearly the expected proportion of the population on the low end of the IQ distribution (16% of the population is expected to be one SD below the mean or lower). The inclusion of individuals with lower IQs in the WLS population is an important sample characteristic, given past research suggesting a possible cognitive phenotype of the premutation of the *FMR1* gene. Reflecting Wisconsin's population in the mid-20th century, the WLS sample is racially and ethnically homogeneous; 99.2% are White and the majority (84.2%) are of Northern European heritage.

In 2008 and 2011, WLS collected saliva samples from participants using Oragene kits (DNA Genotek, Inc., Bethlehem, PA). All participants provided informed consent under a protocol approved by the Institutional Review Board of the University of Wisconsin-Madison. More than two-thirds (69.0%) of surviving WLS members provided saliva samples, and analysis of these samples constitute the basis of the research presented here. Those who provided saliva samples had one-half year more schooling (13.8 years vs. 13.3 years, $p < 0.001$) and three points higher IQ scores (102.2 vs. 98.4, $p < 0.001$) than those who did not return saliva samples. Otherwise, they were representative of the WLS graduate sample as a whole.

Determination of the *FMR1* CGG Triplet Repeat Number

The present study includes 5499 WLS participants (2637 men and 2862 women) for whom saliva samples were obtained and *FMR1* CGG repeats were assayed. DNA was isolated using standard methods. For saliva samples collected in 2008, the number of *FMR1* CGG repeats was determined (under the supervision of author MWB) using a PCR-based protocol that incorporated reagents developed and manufactured by Celera Corporation (Alameda, CA). See Seltzer et al. (11) for details. Additional participants provided saliva samples in 2011; for these samples, repeat number was determined via an assay using the Asuragen AmplideX® Kit (30, 31), conducted in the Rush University Medical Center Molecular Diagnostics Laboratory (supervised by author EB-K). A concordance study was conducted between the two assays. DNA samples initially collected and assayed in 2008 ($n = 22$; some from the premutation range and some with normal alleles) were re-assayed using the Asuragen assay. The correlation between the two was .9996.

For the women, the assays yielded CGG repeat data on the *FMR1* gene on both X chromosomes. Because we did not have activation ratio data, one X chromosome was selected for analysis in the present study as follows. Although we considered alternative approaches (32, 33), we followed the approach of Hunter and colleagues (34). We selected the *longer* allele in women who had one expanded (i.e., > 40 CGGs) and one normal allele ($n = 194$) and in the four cases who had two expanded alleles. Similarly, we selected the *shorter* allele in women who had one low allele (i.e., < 26 CGGs) and one normal allele ($n = 878$) and in women who had two low alleles (n = 139). We randomly

selected one allele for analysis in the present study in women who had two normal alleles (between 26 and 40 CGG repeats, $n = 1589$), and also for those with one low allele and one expanded allele ($n = 58$).

Measurement of Phenotypic Characteristics

All rounds of data collection from the WLS were used to measure the phenotypes evaluated in the present study. In 1957, the source of data was high school records. In 1975, data were collected via a telephone interview. In the 1992 and 2004 rounds of data collection, data were collected via a telephone interview and self-administered questionnaires. In 2011, data were collected via an in-person home visit and self-administered questionnaires. For the present analysis, we evaluated the association between *FMR1* CGG repeat number and indicators of the cognitive, reproductive, and psychiatric phenotypes implicated in prior *FMR1* research.

Cognitive Phenotype

Two measures of cognitive functioning were analyzed. The Henmon-Nelson Test of Mental Abilities (35), was administered when participants were high school students. *IQ scores* were obtained by the WLS from high school records. As noted, participants' scores ranged from 61 (the floor of the test) to 145. See Maenner et al. (36) for details regarding the measurement of IQ in the WLS.

A second measure of cognitive functioning was included, namely *attainment of a college degree*. As all WLS participants included in this study were high school graduates, the measure of educational attainment analyzed for the current research was college graduation. Although most of those who obtained a college degree did so by the 1975 round of data collection, a small number of participants did so subsequently, and thus those who obtained a college degree at any point up to the 2011 round of data collection were included in this analysis.

Reproductive Phenotype

The primary measure of women's fertility was self-reported *age at menopause*. Additionally, we evaluated the *number of biological children born to women*. Women whose ovaries or uterus had been surgically removed ($n = 1,048$) were not included in the analysis of age at menopause. Additionally, among women who had at least one biological child, we used a dichotomous variable indicating whether any of their children had been diagnosed with an *intellectual or developmental disability* (IDD). See Mailick et al. (13) for details describing the determination of disability status in WLS children. All of these measures were obtained in the 2004 round of WLS data collection, when women were age 65.

Psychiatric Phenotype

Symptoms of *depression* were assessed by the Center for Epidemiological Studies-Depression Scale (CES-D) (37), administered at three rounds of WLS data collection (1992, 2004, and 2011 when participants were ages 53, 65, and 72, respectively). For each of 20 depression symptoms, the participant was asked to indicate how many days in the past week the symptom was experienced (0 = never to 3 = 5–7 days; $\alpha = 0.85$). A score of 16 or above was considered an indicator of clinical depression (38, 39). We created a dichotomous variable indicating whether the participant had experienced an episode of clinical depression (i.e., a score of 16 or higher on the CES-D) at any point of data collection.

Data Analysis

CGG repeats in the present cohort ranged from 7 to 128. The next highest number of repeats was 84. Although the case with 128 repeats did not qualify as a statistical outlier as estimated with Cook's Distance (Cook's D), inclusion of the case inflated the standard deviation of the CGG repeat range variable, leading to less precise estimates from the regression models. Therefore, it was decided to focus the present analysis on participants with 84 or fewer CGG repeats. The alpha level was set at .05.

Means and standard deviations of study variables are presented in **Table 1**. Data for college graduation are presented for men only because, whereas men and women had identical IQ metrics, substantially fewer women than men attended college (23.9% vs. 34.2%, chi square = 69.8, $p < 0.001$), reflecting sex-specific differences in expectations for college education in 1957. Thus, college graduation was not considered to be a valid

TABLE 1 | Descriptive statistics of study variables[a].

	Male	Female	Total
CGG repeats	30.1 (5.4) [9, 65] ($n = 2,637$)	selected allele: 29.2 (6.8) [7, 84] ($n = 2,862$) long allele: 32.8 (5.0) [21, 84] short allele: 27.5 (4.4) [7, 43]	29.7 (6.2) [7, 84] ($n = 5,499$)
Birth year	1939.3 (0.52) [1936, 1941] ($n = 2,622$)	1939.5 (0.48) [1930, 1940] ($n = 2,850$)	1939.4 (0.50) [1930, 1941] ($n = 5,472$)
IQ	102.1 (15.2) [61, 145] ($n = 2,637$)	102.3 (14.3) [61, 145] ($n = 2,862$)	102.2 (14.8) [61, 145] ($n = 5,499$)
% college graduate	34.2% ($n = 2,619$)	–	–
Age at menopause	–	50.8 (4.7) [20, 65] ($n = 1,448$)	–
Number of biological children	–	2.84 (1.6) [0, 11] ($n = 2,703$)	–
% having a child with IDD	–	1.4% ($n = 2,438$)	–
% having episodes of clinical depression	17.0% ($n = 2,517$)	24.2% ($n = 2,773$)	20.8% ($n = 5,290$)

[a] *Means, standard deviations (in parentheses), and range (in brackets) are presented unless the variable is marked with (%).*

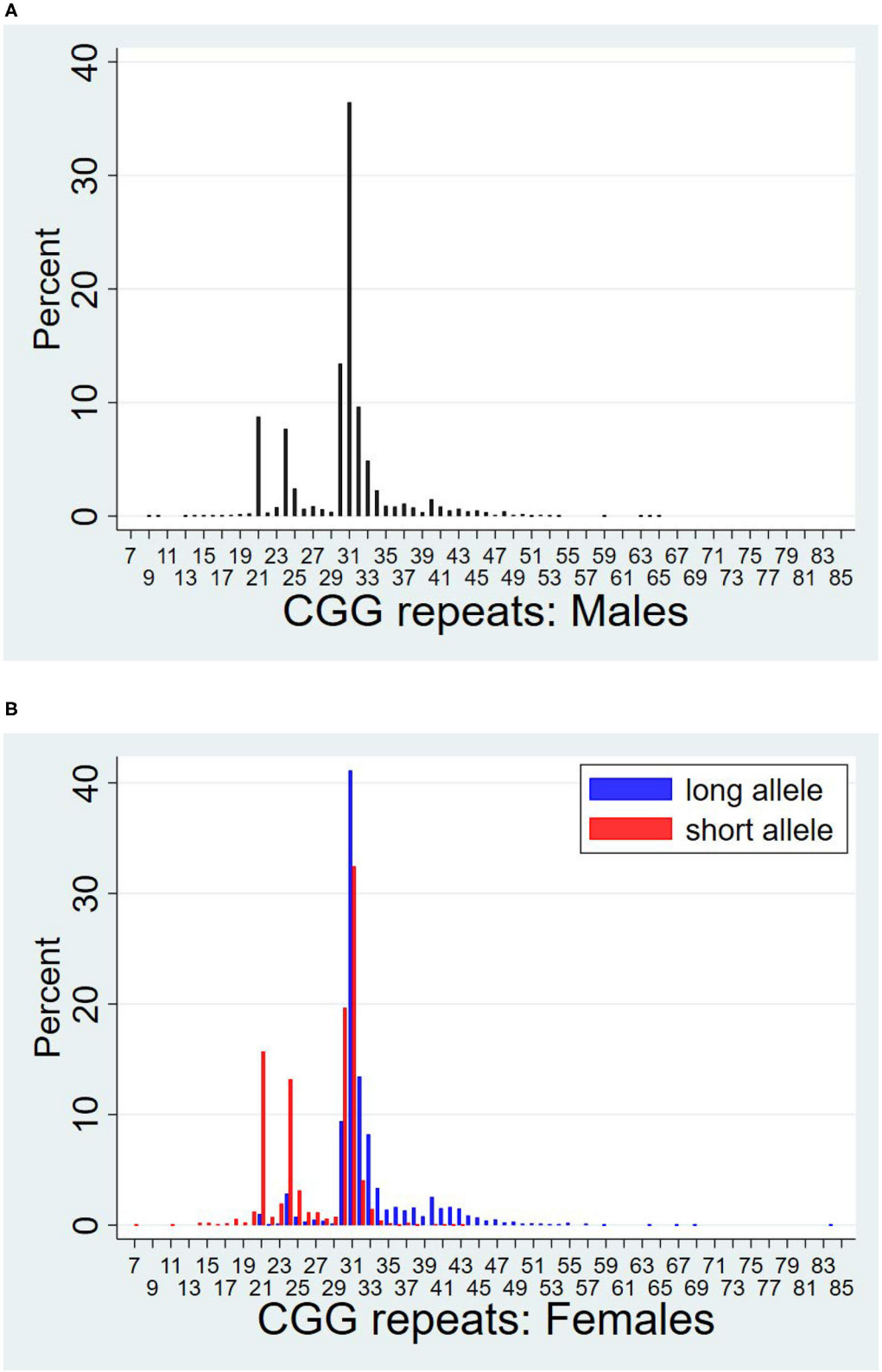

FIGURE 1 | Distributions of CGG repeat lengths by sex. **(A)** Males. **(B)** Females.

measure of cognitive ability for this cohort of women. Data for age at menopause, number of biological children, and whether any child had an IDD diagnosis are presented for women only, given past studies of the association between *FMR1* CGG repeat number and these characteristics, as cited above.

The analytic approach used in this paper treated CGG repeat number as a continuous variable. For the multivariate analyses, Ordinary Least Squares (OLS) and logistic regressions were conducted. For sex-specific analyses (college graduation, age at menopause, number of biological children, having a child with IDD), the regression models included two stages. Model 1 included birth year of the participant as a control variable and CGG repeat number. In Model 2, the squared term of CGG repeats was entered to evaluate curvilinear associations with the dependent variable. For the analysis of IQ and episodes of depression, the regression models included four steps: Models 1 and 2 were the same as above, with the addition of sex of the participant. In Models 3 and 4, interaction terms (CGG repeat X sex, CGG repeat squared X sex) were added to the regression models to evaluate whether the CGG effect differed for men and women. For all analyses, significant CGG effects were graphed showing the scatter plots and the line of best fit between CGG repeat number and the phenotypic variable.

RESULTS

Descriptive Findings

Figure 1 presents a histogram of the distribution of CGG repeats for men (**Figure 1A**) and women (**Figure 1B**). For women, the histogram includes the distributions of both the long and short allele. Women's repeat length on their shorter and longer alleles are also reported in **Table 1**. As shown in **Table 1**, the mean number of repeats for the cohort was 29.7, ranging from 7 to 84 repeats, with men averaging approximately one more repeat than women (30.1 vs. 29.2, $p < 0.001$).

Table 1 also reports the average birth year of study participants, with 1939 being the mean for both men and women. Descriptively, 77% of participants were born in 1939, 12% in 1938, and 9% in 1940. The remaining participants ($n = 5$) were born in earlier or later years.

IQ scores were nearly identical for men and women, averaging 102.2. One-third of the men achieved a college degree. Women averaged 2.84 biological children, ranging from 0 to 11 children. Women who had a natural menopause (i.e., not having surgical removal of ovaries or uterus) averaged 50.7 years of age when they last menstruated (ranging from 20 to 65 years of age). Of women who had at least one biological child, very few (1.4%) reported having a child with a diagnosis of IDD. Women and men differed in their likelihood of having an episode of depression at one or more of the points of data collection when the CES-D was administered, with women being significantly more likely than men (24.2 vs. 17.0%, $p < 0.001$).

Multivariate Findings

Full regression models are presented in **Table 2**. In preliminary analyses of the models including both men and women (IQ, experiencing an episode of depression), terms evaluating the interaction effects of CGG (and CGG squared) with sex were tested. However, neither of these interaction terms reached statistical significance and therefore are not reported in **Table 2**.

TABLE 2 | Associations between CGG repeat length and phenotypes.

	Model 1:	Model 2:
A. IQ[a]		
Birth year	6.55 (0.39)***	6.55 (0.39)***
Sex (female = 1)	−0.707 (0.394)	−0.717 (0.398)
CGG	−0.069 (0.031)*	−0.072 (0.034)*
CGG-squared	–	0.0004 (0.0023)
B. College graduate—males[b]		
Birth year	2.24*** [1.87, 2.68]	2.25*** [1.88, 2.69]
CGG	0.985* [0.969, 0.999]	0.982* [0.967, 0.998]
CGG-squared	–	1.000 [0.999, 1.002]
C. Age at menopause—females[a]		
Birth year	−0.468 (0.278)	−0.459 (0.276)
CGG	−0.014 (0.019)	0.019 (0.021)
CGG-squared	–	−0.004 (0.001)***
D. Number of biological children—females[a]		
Birth year	−0.060 (0.066)	−0.061 (0.066)
CGG	−0.000 (0.005)	−0.006 (0.005)
CGG-squared	–	0.0010 (0.0003)**
E. Having a child with Intellectual or developmental disability—females[b]		
Birth year	0.67 [0.45, 1.00]	0.67 [0.45, 1.00]
CGG	0.981 [0.930, 1.033]	0.978 [0.928, 1.031]
CGG-squared	–	1.001 [0.997, 1.003]
F. Episodes of clinical depression[b]		
Birth year	0.79*** [0.69, 0.90]	0.79*** [0.69, 0.90]
Sex (female = 1)	1.59*** [1.39, 1.83]	1.60*** [1.39, 1.84]
CGG	0.988* [0.977, 0.998]	0.989 [0.978, 1.000]
CGG-squared	–	1.000 [0.999, 1.001]

*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.
[a] *Unstandardized coefficients are presented with standard errors in parenthesis.*
[b] *Odds ratios are presented with 95% confidence intervals in brackets.*

Cognitive Phenotype

As hypothesized, there was a significant negative linear association between CGG repeats and IQ score (see **Table 2A**). **Figure 2A** shows that those who had CGGs at the lower end of the repeat range had IQ scores just above the mean of 100 while those at the higher end of the repeat range scored below the mean. The CGG squared term was not significant.

A similar pattern was observed for college graduation rates among the men, as hypothesized (see **Table 2B**). As shown in **Figure 2B**, the probability of college graduation decreased as CGG repeats increased. Descriptively, nearly 40% of men in the lower end of the CGG distribution were college graduates, whereas at the upper end of the CGG distribution about 25% were college graduates.

Reproductive Phenotype

For the analysis of age at menopause, only women who went through menopause naturally ($n = 1450$) were included

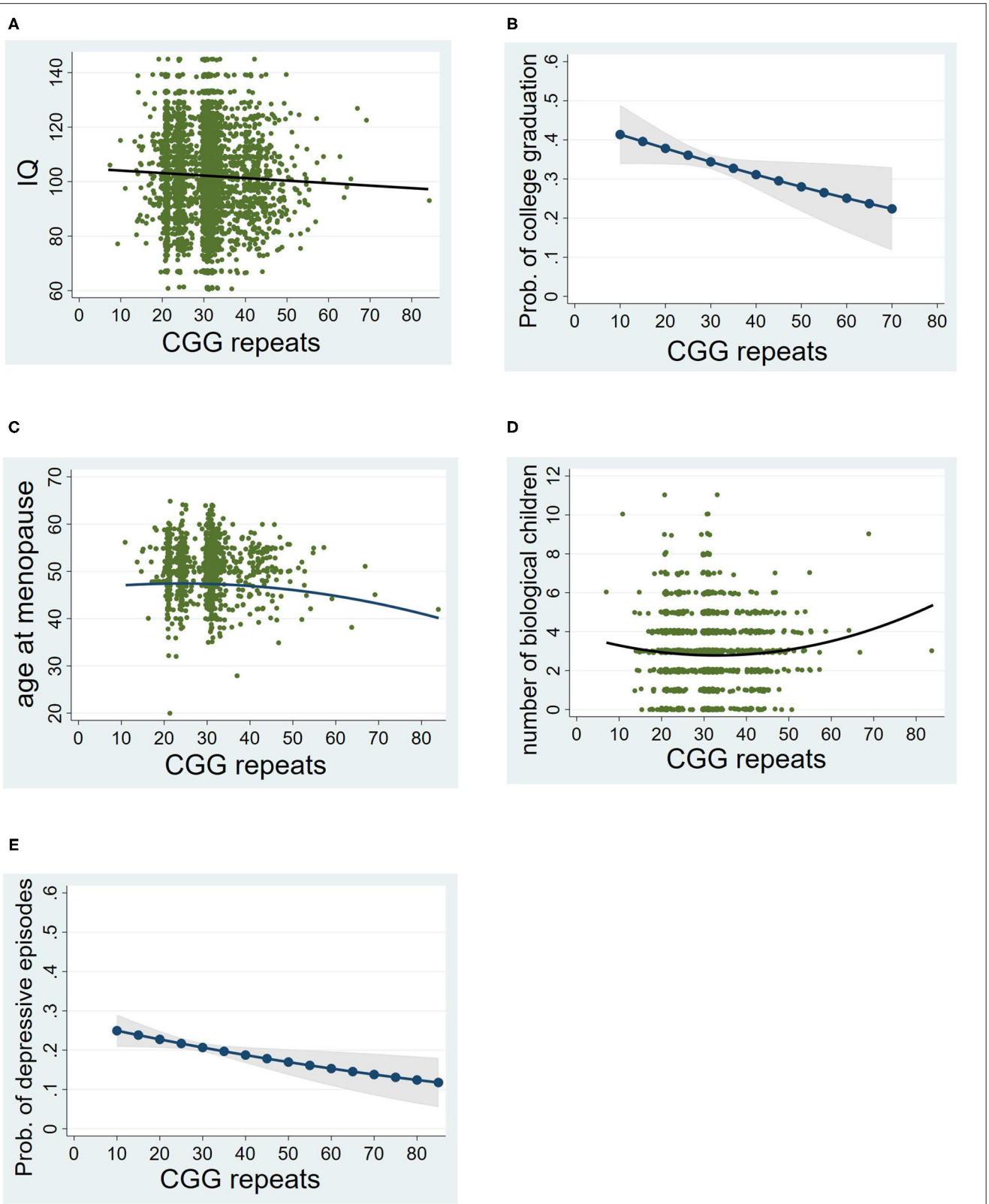

FIGURE 2 | Associations between CGG repeat lengths and phenotypes. **(A)** Linear association with IQ. **(B)** Linear association with college graduate (males only). **(C)** Curvilinear association with age at menopause (females only). **(D)** Curvilinear association with the number of biological children (females only). **(E)** Linear association with episodes of clinical depression. For **(A)**, **(C)**, and **(D)**, a scatterplot with the best fitted line are shown. For **(B)** and **(E)**, predicted probability of the phenotype by CGG repeat lengths are presented; Prob, predicted probability.

(excluding those whose uterus or ovaries were surgically removed). There was a significant curvilinear association between number of CGG repeats and age at menopause (see **Table 2C**). The shape of this association was consistent with the hypothesis. As shown in **Figure 2C**, women at the higher end of the CGG repeat distribution had an earlier age at menopause than those in the middle or lower end of the CGG repeat distribution. Notably, there was no significant association between CGG repeats and the likelihood of having surgical removal of the uterus or ovaries ($p > 0.56$).

There was a significant curvilinear association between CGG repeats and the number of biological children born to the women in the cohort (see **Table 2D**). All women were included in this analysis ($n = 2{,}703$), regardless of whether they went through menopause naturally or due to surgical removal of their uterus or ovaries. Unexpectedly, a *greater* number of children were born to those at the higher end of the CGG repeat distribution than those who were in the middle or lower end of the distribution (see **Figure 2D**), which was counter to our hypothesis. This effect remained significant even after controlling for social factors that might account for larger family size, such as number of marriages and Catholic religious affiliation. Both of these social factors were significant predictors of number of biological children ($p < 0.001$), and the CGG effect nevertheless remained statistically significant even with these factors controlled [regression coefficient (b) = 0.0009, standard error (s.e.) = 0.0003, $p = 0.005$].

In a sensitivity analysis, the association between CGG repeats and number of biological children was re-estimated for the sub-set of women included in the menopause analysis (i.e., those mothers whose menopause was not induced by surgical removal of the uterus or ovaries). Although this sub-set of women was only half as large as those included in the full analysis of number of biological children reported above ($n = 1{,}402$ vs. $n = 2{,}703$), the results were similar [b = 0.007, s.e. = 0.004, $p = 0.057$].

The association between CGG repeat number and whether women had a child with IDD was not statistically significant (see **Table 2E**).

Psychiatric Phenotype

There was a significant negative association between repeat number and the likelihood of exceeding a CES-D score of 16 at any of the three time points of measurement of depression (see **Table 2F**), which was counter to the hypothesis. As descriptively illustrated in **Figure 2E**, more than 20% of those at the lower end of the repeat distribution had at least one episode of depression during adulthood, while just over 10% of those at the upper end of the repeat range had at least one such episode.

DISCUSSION

To the best of our knowledge, the Wisconsin Longitudinal Study offers the first opportunity to evaluate genotype-phenotype associations in a large randomly selected cohort of the U.S. general population, without clinical ascertainment of any cases. The phenotypes evaluated were measures of cognitive functioning, reproductive characteristics, and a psychiatric condition, all selected based on past research on the phenotypes associated with *FMR1* CGG repeat number. There were significant genotype-phenotype associations, but the effects were small in magnitude. The effect of CGG repeats in the range evaluated in this study perhaps is best interpreted as contributing to non-clinical variation in cognitive, reproductive, and psychiatric characteristics in the general population rather than signifying clinical impairment. Nevertheless, understanding how polymorphisms in the *FMR1* gene contribute to "normal" phenotypic variation represents a contribution of the present research. More generally, the inclusion of genetic data in other large NIH-funded population survey studies, such as the MIDUS study (http://midus.wisc.edu/index.php) and Add Health (https://addhealth.cpc.unc.edu/), offers other opportunities for probing such effects of genetic variants.

In some respects, the associations observed in this study were consistent with genotype-phenotype correlations reported in clinical literature on the *FMR1* gene. For example, regarding cognitive functioning, higher numbers of CGG repeats were associated with somewhat lower IQ scores and less likelihood of achieving a college degree (by men), a pattern that points to the more substantial cognitive challenges observed in premutation carriers. Consistent with this observation, in a study of males with normal *FMR1* alleles, the influence of the gene on brain structure and working memory was established, suggesting that the *FMR1* gene is a genetic factor that has implications for the transmission of intelligence even within those who have normal alleles (9). Similarly, the earlier age at menopause among women who had higher numbers of CGG repeats is consistent with much past premutation research (19).

However, other associations were counter to the prediction. Although we hypothesized that women with higher numbers of repeats would report having *fewer* biological children, there was a positive association between repeat number and family size. Follow-up examination of the women in the premutation range in the present cohort who gave birth to their last child after age 30 revealed that they had a larger gap between their last two children (mean = 7.0 years) than other women in the WLS who gave birth to their last child after age 30 (mean gap = 5.0 years). It has been reported that women in the premutation range occasionally move in and out of menopause (19, 40), and it is possible that additional children could have been conceived during the period of skipped cycles. Additionally, as Allen et al. (19) noted, women in the low premutation range in their study (59–79 CGG repeats) were not different from non-carriers with regard to fertility problems or times to first pregnancy. Thus, the patterns observed here, mainly with respect to women in the low premutation range, are not inconsistent with past observations. Future research is needed to parse these effects. There was no association in the present study between repeat number and having a child with an IDD diagnosis, also possibly due to the restriction in the range of CGG repeats at the upper end of the distribution. It is further possible that the WLS population, limited to those who graduated high school, under-represented individuals likely to later give birth to a child with an IDD condition. This might account for the low rate of such children observed here.

The *inverse* association between CGG repeat number and probability of having an episode of depression also was counter to our prediction. Most past studies of the association between CGG repeats and depression were conducted on premutation carrier mothers of children with FXS who had CGG repeats in the upper premutation range or were premutation carriers with FXTAS (3, 25, 41). In these past studies, higher numbers of CGG repeats were associated with depression. In contrast, in the cohort analyzed for the present study, very few participants had children with IDDs and no participant had CGG repeats in the upper premutation range. Nevertheless, the direction of the relationship observed here between CGG repeats and episodes of depression was unexpected, and warrants evaluation in future population research.

The present study is not without limitations. CGG repeats in the present study were below 85 repeats, constraining the detection of phenotypes that could be associated with genotypic variation. Additionally, the WLS cohort lacks racial diversity and thus cannot be generalized to contemporary populations in the U.S. The cohort studied (mainly born in 1939, reaching adulthood in the late 1950s, preceding the baby-boom generation by 6 years) reflects secular trends of that time and as such is not representative of cohorts born in more recent years. Future research is needed to separate secular trends from genetic influences. The lack of measures of AGGs, activation ratio, and FMRP limits the extent of our understanding of genotype-phenotype associations. The findings that were counter to the hypotheses (regarding number of children, having a child with IDD, and episodes of depression) warrant investigation in future research; it is possible that these patterns were due to idiosyncratic characteristics of the WLS cohort. Juxtaposed against these limitations is the unique opportunity offered by the Wisconsin Longitudinal Study to evaluate a large unselected random sample of an age cohort for purposes of exploring phenotypic effects of *FMR1* CGG repeat number.

To conclude, we return to our question of whether *FMR1* CGG repeat number polymorphism is associated with phenotypic variability in the general population. Our data suggest that variation in CGG repeats in this gene contributes systematically—even if not clinically—to population characteristics, particularly with respect to cognitive and reproductive functioning. This study illustrates how research inspired by a rare genetic condition (such as fragile X syndrome) can lead to insights about genotype-phenotype associations in the general population.

DATA AVAILABILITY STATEMENT

The datasets analyzed for this study can be found at https://ssc.wisc.edu/wlsresearch/data/. This study was not preregistered.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of Wisconsin—Madison Institutional Review Board. The participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

JH and MM designed the study and drafted the manuscript. JH performed the data analysis. MWB and EB-K carried out the assays. LD, MWB, and EB-K provided critical reviews. All authors contributed to and approved the manuscript.

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REFERENCES

1. Darnell JC, Van Driesche SJ, Zhang C, Hung KYS, Mele A, Fraser CE, et al. FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell.* (2011) 146:247–61. doi: 10.1016/j.cell.2011.06.013
2. Hall DA. In the gray zone in the fragile X gene: what are the key unanswered clinical and biological questions? *Tremor Other Hyperkinet Mov.* (2014) 4:208 doi: 10.5334/tohm.233
3. Wheeler A, Raspa M, Hagerman R, Mailick M, Riley C. Implications of the FMR1 premutation for children, adolescents, adults, and their families. *Pediatrics.* (2017) 139:S172 LP–S182. doi: 10.1542/peds.2016-1159D
4. Fu Y-H, Kuhl DPA, Pizzuti A, Pieretti M, Sutcliffe JS, Richards S, et al. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. *Cell.* (1991) 67:1047–58. doi: 10.1016/0092-8674(91)90283-5
5. Eichler EE, Hammond HA, Macpherson JN, Ward PA, Nelson DL. Population survey of the human FMR1 CGG repeat substructure suggests biased polarity for the loss of AGG interruptions. *Hum Mol Genet.* (1995) 4:2199–08. doi: 10.1093/hmg/4.12.2199
6. Brown WT, Houck GE, Jeziorowska A, Levinson FN, Ding X, Dobkin C, et al. Rapid fragile X carrier screening and prenatal diagnosis using a nonradioactive PCR test.

JAMA. (1993) 270:1569–75. doi: 10.1001/jama.1993.03510130075034
7. Chen L-S, Tassone F, Sahota P, Hagerman PJ. The (CGG) n repeat element within the 5′ untranslated region of the FMR1 message provides both positive and negative cis effects on in vivo translation of a downstream reporter. *Hum Mol Genet.* (2003) 12:3067–74. doi: 10.1093/hmg/ddg331
8. Snow K, Doud LK, Hagerman R, Pergolizzi RG, Erster SH, Thibodeau SN. Analysis of a CGG sequence at the FMR-1 locus in fragile X families and in the general population. *Am J Hum Genet.* (1993) 53:1217.
9. Wang JY, Hessl D, Iwahashi C, Cheung K, Schneider A, Hagerman RJ, et al. Influence of the fragile X mental retardation (FMR1) gene on the brain and working memory in men with normal FMR1 alleles. *Neuroimage.* (2013) 65:288–98. doi: 10.1016/j.neuroimage.2012.09.075
10. Herd P, Carr D, Roan C. Cohort profile: Wisconsin longitudinal study (WLS). *Int J Epidemiol.* (2014) 43:34–41. doi: 10.1093/ije/dys194
11. Seltzer MM, Baker MW, Hong J, Maenner M, Greenberg J, Mandel D. Prevalence of CGG expansions of the FMR1 gene in a US population-based sample. *Am J Med Genet Part B Neuropsychiatr Genet.* (2012) 159B:589–97. doi: 10.1002/ajmg.b.32065
12. Mailick MR, Hong J, Rathouz P, Baker MW, Greenberg JS, Smith L, et al. Low-normal FMR1 CGG repeat length: Phenotypic associations. *Front Genet.* (2014) 5:309. doi: 10.3389/fgene.2014.00309
13. Mailick M, Hong J, Greenberg J, Dawalt LS, Baker MW, Rathouz PJ. FMR1 genotype interacts with parenting stress to shape health and functional abilities in older age. *Am J Med Genet Part B Neuropsychiatr Genet.* (2017) 174:399–412. doi: 10.1002/ajmg.b.32529
14. Allen EG, Charen K, Hipp HS, Shubeck L, Amin A, He W, et al. Clustering of comorbid conditions among women who carry an FMR1 premutation. *Genet Med.* (2020) 22:758–66. doi: 10.1038/s41436-019-0733-5
15. Klusek J, Porter A, Abbeduto L, Adayev T, Tassone F, Mailick MR, et al. Curvilinear association between language disfluency and FMR1 CGG repeat size across the normal, intermediate, and premutation range. *Front Genet.* (2018) 9:344. doi: 10.3389/fgene.2018.00344
16. Weghofer A, Tea M-K, Barad DH, Kim A, Singer CF, Wagner K, et al. BRCA1/2 Mutations appear embryo-lethal unless rescued by low (CGG n<26) FMR1 sub-genotypes: explanation for the "BRCA paradox"? *PLoS ONE.* (2012) 7:e44753. doi: 10.1371/journal.pone.0044753
17. Grigsby J, Brega AG, Engle K, Leehey MA, Hagerman RJ, Tassone F, et al. Cognitive profile of fragile X premutation carriers with and without fragile X-associated tremor/ataxia syndrome. *Neuropsychology.* (2008) 22:48–60. doi: 10.1037/0894-4105.22.1.48
18. Kraan CM, Hocking DR, Georgiou-Karistianis N, Metcalfe SA, Archibald AD, Fielding J, et al. Impaired response inhibition is associated with self-reported symptoms of depression, anxiety, and ADHD in female FMR1 premutation carriers. *Am J Med Genet Part B Neuropsychiatr Genet.* (2014) 165:41–51. doi: 10.1002/ajmg.b.32203
19. Allen EG, Sullivan AK, Marcus M, Small C, Dominguez C, Epstein MP, et al. Examination of reproductive aging milestones among women who carry the FMR1premutation. *Hum Reprod.* (2007) 22:2142–52. doi: 10.1093/humrep/dem148
20. Mailick MR, Hong J, Greenberg J, Smith L, Sherman S. Curvilinear association of CGG repeats and age at menopause in women with FMR1 premutation expansions. *Am J Med Genet Part B Neuropsychiatr Genet.* (2014) 165:705–11. doi: 10.1002/ajmg.b.32277
21. Movaghar A, Page D, Brilliant M, Baker MW, Greenberg J, Hong J, et al. Data-driven phenotype discovery of FMR1 premutation carriers in a population-based sample. *Sci Adv.* (2019) 5:eaaw7195. doi: 10.1126/sciadv.aaw7195
22. Sherman SL, Taylor K, Allen EG. FMR1 premutation: a leading cause of inherited ovarian dysfunction. In: *Fragile Sites: New Discoveries and Changing Perspectives*. Hauppauge: Nova Science Publishers, Inc. (2007). p. 229–320.
23. Spath MA, Feuth TB, Smits APT, Yntema HG, Braat DDM, Thomas CMG, et al. Predictors and risk model development for menopausal age in fragile X premutation carriers. *Genet Med.* (2011) 13:643–50. doi: 10.1097/GIM.0b013e31821705e5
24. Nolin SL, Glicksman A, Ersalesi N, Dobkin C, Brown WT, Cao RU, et al. Fragile X full mutation expansions are inhibited by one or more AGG interruptions in premutation carriers. *Genet Med.* (2015) 17:358–64. doi: 10.1038/gim.2014.106
25. Roberts JE, Bailey DB, Mankowski J, Ford A, Sideris J, Weisenfeld LA, et al. Mood and anxiety disorders in females with the FMR1 premutation. *Am J Med Genet Part B Neuropsychiatr Genet.* (2009) 150:130–9. doi: 10.1002/ajmg.b.30786
26. Klusek J, Hong J, Sterling A, Berry-Kravis E, Mailick MR. Inhibition deficits are modulated by age and CGG repeat length in carriers of the FMR1 premutation allele who are mothers of children with fragile X syndrome. *Brain Cogn.* (2020) 139:105511. doi: 10.1016/j.bandc.2019.105511
27. Seltzer MM, Barker ET, Greenberg JS, Hong J, Coe C, Almeida D. Differential sensitivity to life stress in FMR1 premutation carrier mothers of children with fragile X syndrome. *Heal Psychol.* (2012) 31:612–22. doi: 10.1037/a0026528
28. Leehey MA, Berry-Kravis E, Goetz CG, Zhang L, Hall DA, Li L, et al. FMR1 CGG repeat length predicts motor dysfunction in premutation carriers. *Neurology.* (2008) 70:1397–402. doi: 10.1212/01.wnl.0000281692.98200.f5
29. Tassone F, Adams J, Berry-Kravis EM, Cohen SS, Brusco A, Leehey MA, et al. CGG repeat length correlates with age of onset of motor signs of the fragile X-associated tremor/ataxia syndrome (FXTAS). *Am J Med Genet Part B Neuropsychiatr Genet.* (2007) 144:566–9. doi: 10.1002/ajmg.b.30482
30. Chen L, Hadd A, Sah S, Filipovic-Sadic S, Krosting J, Sekinger E, et al. An information-rich CGG repeat primed PCR that detects the full range of fragile X expanded alleles and minimizes the need for southern blot analysis. *J Mol Diagnostics.* (2010) 12:589–600. doi: 10.2353/jmoldx.2010.090227
31. Grasso M, Boon EMJ, Filipovic-Sadic S, Van Bunderen PA, Gennaro E, Cao R, et al. A novel methylation PCR that offers standardized determination of FMR1 methylation and CGG repeat length without southern blot analysis. *J Mol Diagn.* (2014) 16:23–31. doi: 10.1016/j.jmoldx.2013.09.004
32. Kline JK, Kinney AM, Levin B, Brown SA, Hadd AG, Warburton D. Intermediate CGG repeat length at the FMR1 locus is not associated with hormonal indicators of ovarian age. *Menopause.* (2014) 21:740–8. doi: 10.1097/GME.0000000000000139
33. Gustin SLF, Ding VY, Desai M, Leader B, Baker VL. Evidence of an age-related correlation of ovarian reserve and FMR1 repeat number among women with "normal" CGG repeat status. *J Assist Reprod Genet.* (2015) 32:1669–76. doi: 10.1007/s10815-015-0577-0
34. Hunter JE, Leslie M, Novak G, Hamilton D, Shubeck L, Charen K, et al. Depression and anxiety symptoms among women who carry the FMR1 premutation: impact of raising a child with fragile X syndrome is moderated by CRHR1 polymorphisms. *Am J Med Genet B Neuropsychiatr Genet.* (2012) 159B:549–59. doi: 10.1002/ajmg.b.32061
35. Henmon VAC, Nelson MJ. The measurement of intelligence. educational progress bulletin 13. (1937).
36. Maenner MJ, Greenberg JS, Mailick MR. Association between low IQ scores and early mortality in men and women: evidence from a population-based cohort study. *Am J Intellect Dev Disabil.* (2015) 120:244–57. doi: 10.1352/1944-7558-120.3.244
37. Radloff LS. The CES-D scale: a self-report depression scale for research in the general population. *Appl Psychol Meas.* (1977) 1:385–401. doi: 10.1177/014662167700100306
38. Lewinsohn PM, Seeley JR, Roberts RE, Allen NB. Center for Epidemiologic Studies Depression Scale (CES-D) as a screening instrument for depression among community-residing older adults. *Psychol Aging.* (1997) 12:277. doi: 10.1037/0882-7974.12.2.277
39. Weissman MM, Sholomskas D, Pottenger M, Prusoff BA, Locke BZ. Assessing depressive symptoms in five psychiatric populations: a validation study. *Am J Epidemiol.* (1977) 106:203–14. doi: 10.1093/oxfordjournals.aje.a112455
40. Wittenberger MD, Hagerman RJ, Sherman SL, McConkie-Rosell A, Welt CK, Rebar RW, et al. The FMR1 premutation and reproduction. *Fertil Steril.* (2007) 87:456–65. doi: 10.1016/j.fertnstert.2006.09.004
41. Hagerman R, Hagerman P. Advances in clinical and molecular understanding of the FMR1 premutation and fragile X-associated tremor/ataxia syndrome. *Lancet Neurol.* (2013) 12:786–98. doi: 10.1016/S1474-4422(13)70125-X

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Metabolomic Biomarkers Are Associated With Area of the Pons in Fragile X Premutation Carriers at Risk for Developing FXTAS

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Fragile X-associated tremor/ataxia syndrome (FXTAS) is a late adult-onset neurodegenerative disorder that affects movement and cognition in male and female carriers of a premutation allele (55–200 CGG repeats; PM) in the fragile X mental retardation (*FMR1*) gene. It is currently unknown how the observed brain changes are associated with metabolic signatures in individuals who develop the disorder over time. The primary objective of this study was to investigate the correlation between longitudinal changes in the brain (area of the pons, midbrain, and MCP width) and the changes in the expression level of metabolic biomarkers of early diagnosis and progression of FXTAS in PM who, as part of an ongoing longitudinal study, emerged into two distinct categories. These included those who developed symptoms of FXTAS (converters, CON) at subsequent visits and those who did not meet the criteria of diagnosis (non-converters, NCON) and were compared to age-matched healthy controls (HC). We assessed CGG repeat allele size by Southern Blot and PCR analysis. Magnetic Resonance Imaging (MRIs) acquisition was obtained on a 3T Siemens Trio scanner and metabolomic profile was obtained by ultra-performance liquid chromatography, accurate mass spectrometer, and an Orbitrap mass analyzer. Our findings indicate that differential metabolite levels are linked with the area of the pons between healthy control and premutation groups. More specifically, we observed a significant association of ceramides and mannonate metabolites with a decreased area of the pons, both at visit 1 (V1) and visit 2 (V2) only in the CON as compared to the NCON group suggesting their potential role in the development of the disorder. In addition, we found a significant correlation of these metabolic signatures with the FXTAS stage at V2 indicating their contribution to the progression and pathogenesis of FXTAS. Interestingly, these metabolites, as part of lipid and sphingolipid lipids pathways, provide evidence of the role that their dysregulation plays in the development of FXTAS and inform us as potential targets for personalized therapeutic development.

Keywords: fragile X-associated tremor/ataxia syndrome, area of the pons, metabolic biomarkers, brain measures, lipids, premutation carriers

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INTRODUCTION

Aging is a complex and evolutionarily conserved process that is found to be one of the main risk factors for a number of human neurodegenerative disorders (1). Aging and many aging-associated disorders share a range of molecular or cellular pathologies, which can involve a dysregulated energy balance. Increasing evidence suggests that metabolic alterations can strongly influence the development and the progression of various neurodegenerative disorders. Although the brain represents only 2% of the total body weight, it accounts for 20% of an individual's energy expenditure at rest (2). Thus, compromised energy metabolism and adverse changes, are potentially contributing to increased vulnerability of the brain to develop neurodevelopmental and neurodegenerative processes (3).

Fragile X-associated Tremor/Ataxia Syndrome (FXTAS) is a late-onset neurodegenerative disorder, mostly affecting carriers of the fragile X mental retardation 1 (*FMR1*) gene mutation after the age of 50. Currently, there is no effective treatment for FXTAS, and the cognitive and/or motor symptoms progressively worsen over time, causing reduced quality of life, increased medical costs, and eventually, death. FXTAS is caused by the expanded CGG repeats (55–200 CGG) within the 5′UTR of the *FMR1* gene. In normal healthy individuals, the number of CGG repeats lies between 5 and 54 while individuals carrying alleles with a CGG repeat expansion >200 develop fragile X syndrome (FXS), the most common form of intellectual disability and known monogenic cause of autism spectrum disorders (ASD) (4). The high prevalence of the premutation allele among the general population (1:110–200 females and 1:430 males), leads to an estimate of approximately 1.5 million individuals in the general US population being at risk for *FMR1* associated disorders, over their life spans. In addition, among the PM population, an estimated 40–75% of male and 8–16% of female PMs are at risk of developing FXTAS (5, 6).

FXTAS core features include progressive intention tremor and cerebellar gait ataxia, autonomic dysfunction, and parkinsonism. Neuropathologically, it is characterized by the presence of ubiquitin-positive intranuclear inclusions in neurons and astrocytes throughout the brain and in Purkinje cells (7). In addition to the clinical and neuropathological features, the radiological signs, including white matter hyperintensities (wmhs) in the middle cerebellar peduncles (the "MCP sign") (8) also contribute to the diagnosis of FXTAS. Similarly, a significant prevalence of wmhs in the splenium of the corpus callosum (9, 10), generalized brain atrophy, increased T2 signal in area of the pons and periventricular regions along with the subcortical gray matter damage with atrophy of the midbrain, are part of the pathogenesis of FXTAS (5, 11).

The brainstem is the central axis of the brain and both of its regions, the area of the pons and the midbrain, play an important role in sensation and movement (12). The upper area of the pons and midbrain tegmentum are the main components of the ascending reticular activating system and associated with various other neurodegenerative disorders (13). Measurements of these areas have been shown previously to successfully differentiate subcortical movement disorders, such as Parkinson's disease (14), which presents with resting tremor that has also been observed in FXTAS. In addition, middle cerebellar peduncle (MCP) width showed a great sensitivity and specificity in differentiating multiple system atrophy from other disorders (15). We recently reported the MCP width as novel biomarker for FXTAS (16); decreased MCP width was observed in individuals who later developed symptoms of FXTAS as compared to premutation carriers (PM) who did not, and healthy controls. In addition, we also found reduced midbrain and area of the pons cross-sectional areas in patients with FXTAS compared to PM without FXTAS and controls (16). In a more recent study, we reported the association between these brain measures, including reduced MCP and SCP width, midbrain, and area of the pons cross-sectional area with increased expression levels of the *Iso10/10b, Iso4/4b FMR1* mRNA isoforms of the *ASFMR1* 131 bp mRNA isoform (17), suggesting their potential role in the pathogenesis of FXTAS.

Metabolic alterations and mitochondrial dysfunction have been extensively investigated in numerous age-related neurodegenerative disorders (18). However, the relationships between systemic abnormalities in metabolism and the pathogenesis of FXTAS are poorly understood. Previous metabolomic studies have investigated a panel of four core serum metabolites (phenethylamine (PEA), oleamide, aconitate, and isocitrate) for sensitive and specific diagnosis of the PM with and without FXTAS and found oleamide/isocitrate as a biomarker of FXTAS (19). Later, mitochondrial dysfunction, markers of neurodegeneration, and pro-inflammatory damage in PM were reported (20). Increased mitochondrial oxidative stress in primary fibroblasts derived from PM, compared with age and sex-matched controls has also been observed (21). Napoli and colleagues found the presence of the Warburg effect (which involves an increase in the rate of glucose uptake and preferential production of lactate, even in the presence of oxygen) in the peripheral blood mononuclear cells (PBMCs)'s derived from the controls, in PM with and without FXTAS (22). Later, Napoli et al. observed a significant impact of allopregnanolone treatment on oxidative stress, GABA metabolism, and mitochondria-related outcomes, and suggested allopregnanolone as a potential therapeutic for the cognitive and GABA metabolism improvement in FXTAS patients (23). In the premutation animal model's significant metabolic changes were found in the sphingolipid and purine metabolism in the cerebellum of premutation mice while the *Schlank (Cers5)*, *Sk2 (Sphk1),* and *Ras (Impdh1)* genes were suggested as genetic modifiers of CGG toxicity in Drosophila (24). It is, however, unclear how global perturbations in metabolism may be related to severity of FXTAS pathology and the eventual expression of symptoms in individuals at risk for developing FXTAS. Our recent study identified metabolic biomarkers of FXTAS early diagnosis and disease progression by characterizing individuals who developed symptoms of FXTAS over time. Specifically, we found that lipid metabolism and specifically the sub pathways involved in mitochondrial bioenergetics, are significantly altered in FXTAS (25).

To date, no study evaluating the metabolic alterations in correlation with brain changes in PM who develop symptoms of FXTAS over time has been reported. In the current study, we evaluated male participants, carriers of the *FMR1* premutation allele, enrolled in an ongoing longitudinal study carried out at the UC Davis MIND Institute. The participants were followed for at least two longitudinal time points (Visit 1, V1, and Visit 2, V2) during which neuroimaging, neuropsychological, molecular measurements, as well as medical and neurological examinations were collected. A subset of the premutation participants, all symptom-free at the time of enrollment, developed symptoms that warranted a diagnosis of FXTAS by Visit 2. We define these individuals as converters (CON). The remaining premutation participants, who did not develop symptoms of FXTAS by Visit 2, we define as non-converters (NCON). In the current work, we investigated whether the expression levels of identified metabolic biomarkers were associated with changes in brain measures including the midbrain and pons cross-sectional area and MCP width, in the CON group compared to the NCON and HC groups. In addition, we also investigated the association of metabolite expression with the progression of FXTAS. Understanding the metabolic variations along with brain changes in PM who developed FXTAS symptoms over time is likely to provide insights into novel disease-modifying treatments for this progressive neurodegenerative disorder.

MATERIALS AND METHODS

Study Participants

As part of a continuing longitudinal study at UC Davis MIND Institute, male PM, >45 years of age, and non-carrier age-matched controls were recruited from throughout the USA and Canada [as detailed in (16)]. All male participants were white in race; there were three Hispanic participants in the HC group, one in the CON group, and zero in NCON group. The studies and all protocols were carried out in accordance with the Institutional Review Board at the University of California, Davis. All participants gave written informed consent before participating in the study in line with the Declaration of Helsinki. FXTAS stage scoring was based on the clinical descriptions as previously described (26). Three categories were used in the diagnosis of FXTAS as explained in Zafarullah and Tassone (27) and termed as "definite," "probable" and "possible." Three age-matched groups were included in this study: CON, NCON, and HC. Using the data from two brain scans, from neurological assessment, FXTAS stage, and CGG repeat length, 10 participants were classified as "CON" as they developed clear FXTAS symptomology between visits (FXTAS stage score was 0–1 at V1 and ≥2 at V2); 10 were defined as "NCON" because they continued to show no signs of FXTAS at V2 (FXTAS stage score was 0–1 at both V1 and V2) and 10 as HC (normal *FMR1* alleles/non-PM).

CGG Repeat Length

Genomic DNA (gDNA) was isolated from 5 mL of peripheral blood leukocytes using the Gentra Puregene Blood Kit (Qiagen). CGG repeat allele size and methylation status were assessed by using the combination of Southern Blot and PCR analysis as previously reported (28, 29).

Brain Measures

The following methods including MRI acquisition and MRPI analysis were originally described in our previous report (16). High resolution structural magnetic resonance imaging (MRIs) acquisition was obtained on a 3T Siemens Trio scanner using a 32-channel head coil and a T1-weighted 3D MPRAGE sequence with the following parameters: $TR = 2{,}170$ ms, $TE = 4.86$ ms, flip angle $= 7^{\circ}$, FoV = 256 mm2, 192 slices, 1 mm slice thickness. The scans were first aligned along the anterior-posterior commissure line using acpc detect (http://www.nitrc.org/projects/art) (30) or manually using DTI Studio (www.mristudio.org) (31). Then MRI bias field correction was performed using N4 (http://stnava.github.io/ANTs/) (32). A series of independent raters (two per measure) who were blinded to the participant age, group, and time point, quantitatively assessed all MR images for four measurements of brain morphology: MCP width as well pons and midbrain cross-sectional areas were based on methods previously described (33, 34).

Sample Preparation and Metabolite Profiling

Plasma metabolite profiling was determined by a non-targeted platform that allows the relative quantitative analysis of a large number of molecules (35). Samples were stored at −80°C until processing and then prepared using the automated MicroLab STAR® (Hamilton Company, Reno, NV, USA). Several recovery standards were added prior to the first step in the extraction process for QC purposes. To remove protein, dissociate small molecules bound to protein or trapped in the precipitated protein matrix, and to recover chemically diverse metabolites, proteins were precipitated with methanol under vigorous shaking for 2 min (Glen Mills GenoGrinder 2000) followed by centrifugation. The resulting extract was divided into five fractions: two for analysis by two separate reverse phases (RP)/UPLC-MS/MS methods with positive ion mode electrospray ionization (ESI), one for analysis by RP/UPLC-MS/MS with negative ion mode ESI, one for analysis by HILIC/UPLC-MS/MS with negative ion mode ESI, and one sample were reserved for backup. Samples were placed briefly on a TurboVap® (Zymark) to remove the organic solvent. The sample extracts were stored overnight under nitrogen before preparation for further analysis as explained in Zafarullah et al. (25).

Statistical Analysis

The association between brain measures and metabolites at a single visit was analyzed using linear regression models that included a brain measure as the area of the pons and a metabolite as the single covariate. The association between changes in brain measures and in metabolites between visits was analyzed using linear regression models that included change in a brain measure as the area of the pons and change in metabolite, baseline metabolite level, and baseline brain measure as covariates. Models fitted to visit 1 data included all subjects (control, NCON, and CON), and models fitted to visit 2 data included all

TABLE 1 | Demographic information on age and CGG repeats in three male participant groups: HC, CON and NCON.

		Healthy controls (HC)	Converters (CON)	Non-converters (NCON)	All patients	*P*-Value (*F*-Test)
Age	N	10	10	10	30	0.936
	Mean (SD)	65.60 (3.239)	63.50 (6.786)	63.20 (4.849)	64.10 (5.101)	
	Median (Range)	64.50 (62–70)	63.50 (53–75)	64.00 (52–69)	64.00 (62–75)	
CGG	N	10	10	10	30	<0.001
	Mean (SD)	28.90 (4.095)	93.30 (22.91)	75.70 (18.73)	65.97 (32.26)	
	Median (Range)	30 (20–32)	84.50 (74–141)	74 (56–122)	72 (20–141)	

premutation subjects (NCON and CON). Specifically, all the Visit 1 regression analyses included all subjects ($n = 30$), and all the Visit 2 regression analyses included all premutation subjects ($n = 20$). *P*-values were adjusted for multiple testing (within each analysis, across metabolites) using the Benjamini-Hochberg false discovery rate controlling method (36). Analyses were conducted using R version 4.0.2 (37).

RESULTS

Demographics

Three groups of male participants were included in this study: 1) PM who converted at V2 (CON; $n = 10$), 2) PM who did not convert at V2 (NCON; $n = 10$) and 3) healthy controls (HC; $n = 10$). All participants in the CON and NCOV groups were matched for age and CGG repeat length as reported in **Table 1**. Participant race, age, and ethnicity did not differ significantly between the three groups. As expected, CGG repeat size was significantly lower in healthy controls than in the CON and NCON groups ($P < 0.001$ in both comparisons) but it was not significantly different between the two premutation carrier groups of CON and NCON ($P = 0.76$).

Differential Metabolite Levels Linked With Area of the Pons Area in Healthy Control and Premutation Groups

We have recently reported 94 potential metabolic biomarkers for early diagnosis and progression of FXTAS that showed significant changes in expression ($P \leq 0.05$) in the CON as compared to the NCON both at V1 and V2 or only at V2 (25). In this study, we investigated the correlation between these potential metabolic biomarkers and brain measures (midbrain, area of the pons, and MCP width) among healthy control (HC), and PM including converter and non-converter (CON and NCON) at V1. We found a significant association ($P \leq 0.05$) of expression level of six metabolites with area of the pons among all three groups (HC, CON, and NCON) at V1 (**Figure 1**). While no significant correlation of the midbrain and MCP width with the identified metabolites at baseline has been observed.

Expression Levels of Metabolic Biomarkers Associated With Brain Measures

Within the two premutation groups, the levels of 11 metabolites showed a significant correlation ($P \leq 0.05$) with decreased area of the pons at V1 while four showed a significant correlation at V2 only in the CON group but not in the NCON group. Interestingly, level of ceramide (d16:1/24:1, d18:1/22:1) correlated with area of the pons area both at V1 [Regression Slope −72.3 (−118.7, −25.9); *P*-value 0.0496; **Figure 2A**] and V2 [Regression Slope −56.7 (−88.3, −25.2); *P*-value 0.0597; **Figure 2B**]. Similarly, we also observed a significant correlation between mannonate and area of the area of the pons both at V1 [Regression Slope −97.3 (−162.3, −32.3); *P*-value 0.0496; **Figure 2C**] and V2 [Regression Slope −135 (−203.8, −67.2); *P*-value 0.0543; **Figure 2D**]. No significant correlations were observed between the midbrain area and MCP width and any metabolites both at V1 and V2 between CON and NCON premutation groups.

Metabolite Expression Levels Correlate With FXTAS Progression

We evaluated the differential expression of the metabolic biomarkers with the progression of FXTAS and with the FXTAS stage in the CON and NCON participants at V2. We observed that 27 metabolites significantly correlated with change in FXTAS stage from V1 to V2 with the majority of these metabolites being lipids followed by xenobiotics, amino acids, and energy (**Table 2**). Further we observed a significant correlation between the expression levels of several of these metabolites with the FXTAS stage (**Figure 3A**). Interestingly, six of these metabolites including palmitate (16:0) (**Figure 3A**), palmitoylcarnitine (C16), palmitoleate (16:1n7), fumarate, lactosyl-N-behenoyl-sphingosine (d18:1/22:0), and ceramide (d16:1/24:1, d18:1/22:1) have been reported to be critically involved in the development of other neurodegenerative disorders. In addition, these metabolites are part of the lipid and fatty acid metabolism (**Figure 3B**) and sphingolipid metabolism (**Figure 3C**). We previously shown that lipid metabolism was associated with the development and progression of FXTAS (changes of the FXTAS stage from V1 to V2) (25) and this association has also been reported in the premutation mouse model (24).

DISCUSSION

The present study results provide evidence that brain measures, specifically the area of the pons cross-sectional area, correlate with plasma levels of metabolites that are part of the fatty acid

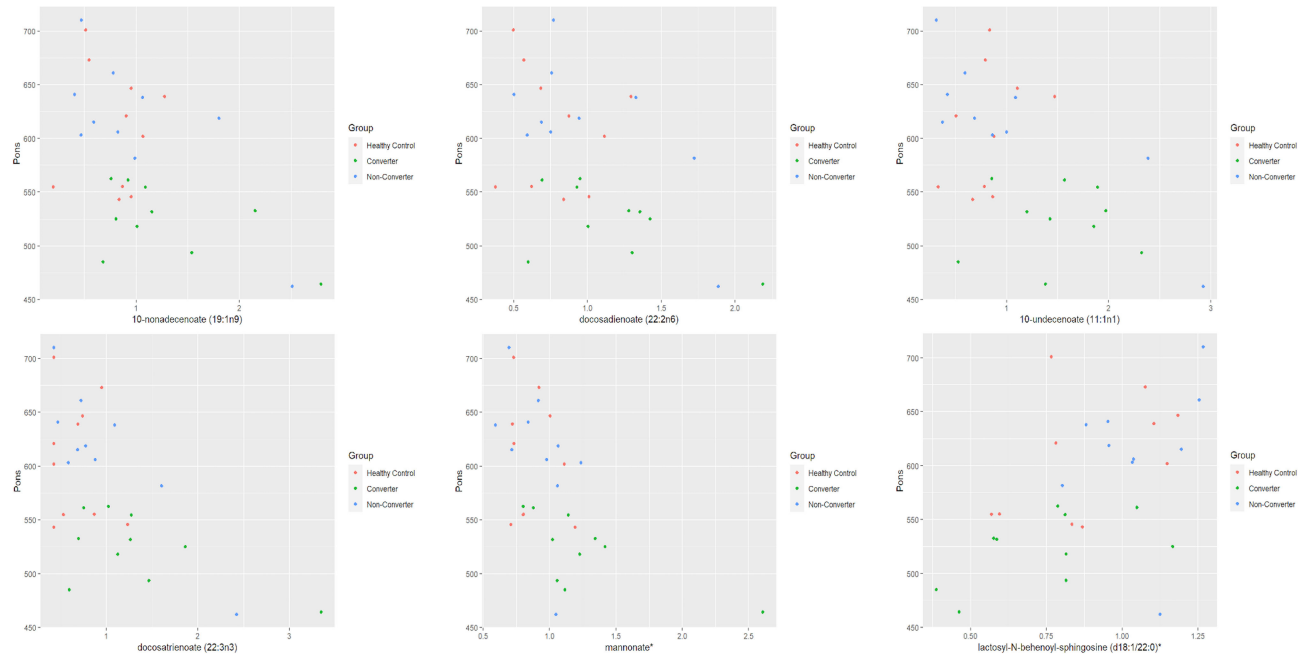

FIGURE 1 | Pons by metabolite expression among HC, CON and NCON. Scatter plots showing correlation between pons and metabolite expression levels. The dots in red are representing the plotted values obtained for HC, green for CONV and blue for NCON. *The identity of these metabolites has not been officially confirmed based on the standard.

and sphingolipid metabolism. These findings expand upon our previous study of plasma metabolic profiling of participants who developed symptoms of FXTAS over time (25), potentially representing biomarkers of early diagnosis and progression of FXTAS and suggest that these factors play a role in the brain structure of individuals with FXTAS.

Magnetic resonance imaging (MRI) takes advantage of a strong magnetic field for non-invasively imaging of parts of the brain parts to identify regional tissue abnormalities and to obtain volumes of brain structures. The imaging profile provides an opportunity to not only visualize the neuroanatomical and functional signatures of various neurodegenerative disorders, but it can also identify disease-specific biomarkers of the underlying processes. Various imaging biomarkers have been reported in Parkinson's disease (PD) (38), Amyotrophic Lateral Sclerosis (ALS) (39), Alzheimer Disease (AD) (40), and Dementia (41) and recently by our team in FXTAS (16).

The brainstem (which includes midbrain, area of the pons, and the medulla oblongata) is a critical regulator of vital bodily functions (42) with midbrain and area of the pons primarily supporting cognition and mood while medulla oblongata regulates cardiovascular and respiratory functions (43). Interestingly, lesions and atrophy of these brainstem structures represent the hallmarks of various neurological disorders and recent findings have pointed to a much deeper involvement of the brainstem nuclei which could change our understanding of the cause, prevalence and early diagnosis of these devastating diseases. Altered volume of midbrain, area of the pons, and medulla oblongata have been reported in individuals with schizophrenia (SCZ), bipolar disorder (BD), multiple sclerosis (MS), dementia, mild cognitive impairment (MCI), and Parkinson's disease (PD) in comparison to healthy controls (HC) (44, 45). Interestingly, reduction in area of the pons over time can significantly discriminate MSA from Progressive Supranuclear Palsy (PSP) (46). Moreover, the Fractional Anisotropy (FA) and Apparent Diffusion Coefficient (ADC) values in the area of the pons can differentiate the middle cerebellar peduncles parkinsonian subtype (MSA-P) patients from PD with 100% specificity (47). Interestingly, the voxel-based morphometry (VBM) analysis has also identified neurodegenerative changes primarily in the midbrain and area of the pons of PSP patients as compared to controls (48). Finally, degeneration of the locus coeruleus (LC), a long and narrow nucleus in the area of the pons, correlates with cognitive dysfunction and potentiate pathology of AD (49).

In our earlier studies, we observed the variation in the MCP width, area of the pons and midbrain cross-sectional areas as well as their significant association with the molecular measures in individuals who developed symptoms of FXTAS over time as compared to non-symptomatic PM and healthy controls, suggesting their role in FXTAS pathogenesis and progression (16, 17). These findings point toward the critical involvement of the area of the pons in neurodegenerative disorders, which could potentially provide information about the neuropathology of the disease and lead to early clinical diagnosis of these diseases.

Metabolomics is the omics platform that measures levels of metabolites in biological samples (50) uncovering potential biomarkers of aging and neurodegenerative diseases such as

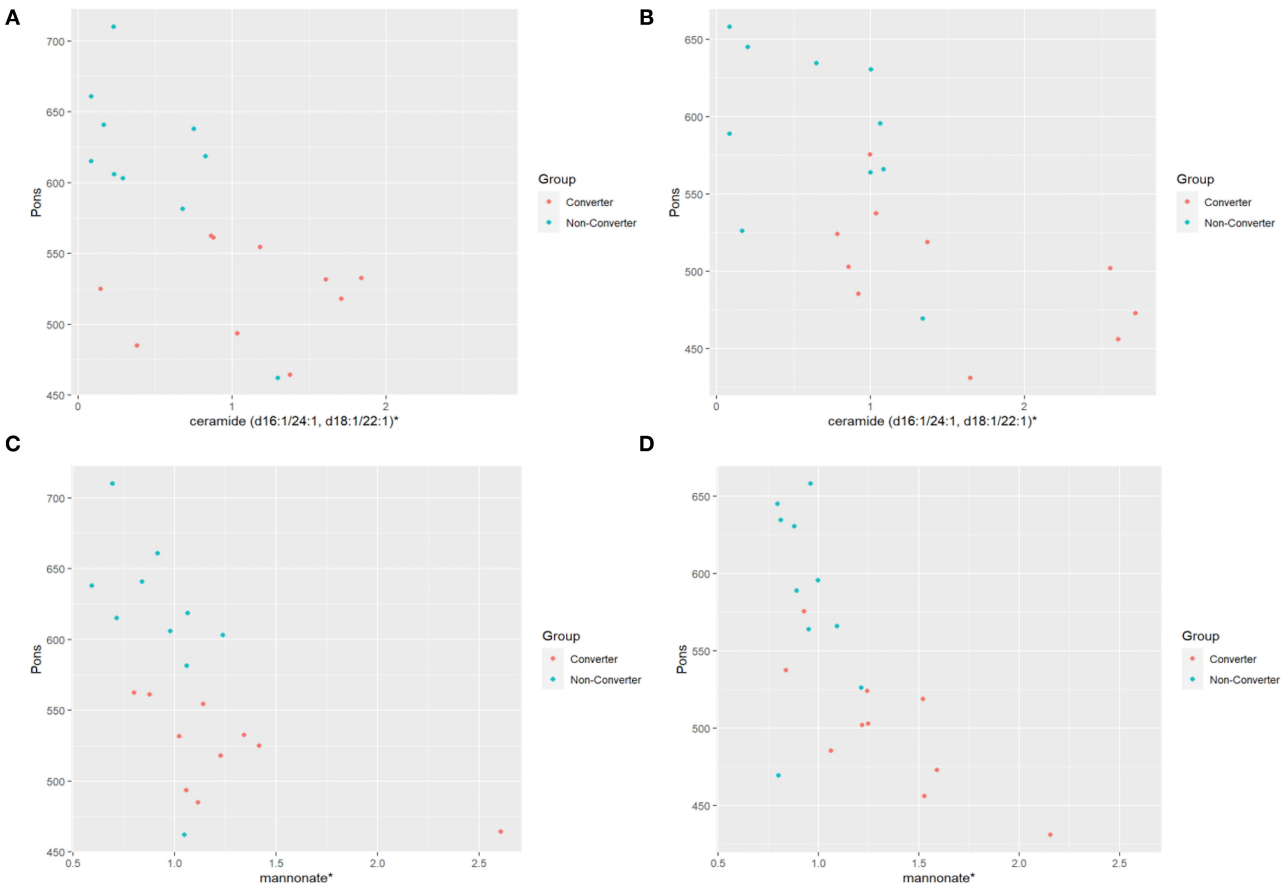

FIGURE 2 | Distribution of metabolic biomarkers with pons between CON and NCON groups. **(A)** Scatter plots showing correlation between pons and ceramide in CON and NCON at V1. The dots in red are representing the plotted values obtained for CON, and turquoise for NCON. **(B)** Scatter plots showing correlation between pons and ceramide in CON and NCON at V2. **(C)** Scatter plots showing correlation between pons and mannonate in CON and NCON at V1. **(D)** Scatter plots showing correlation between pons and mannonate in CON and NCON at V2. *The identity of these metabolites has not been officially confirmed based on the standard.

AD (51), Parkinson (52), Huntington (53), MS (54), and Amyotrophic Lateral Sclerosis (55) and FXTAS (25). A larger number of untargeted metabolomics-based studies have been reported using plasma/serum samples, due to its minimally invasive nature and relatively easy availability of blood samples. Unique metabolic signatures associated with altered energy homeostasis, Krebs cycle, changes in lipid membrane associated with abnormal CSF Aβ42 levels, altered mitochondrial function, neurotransmitter and lipid biosynthesis, are altered in plasma of patients with mild cognitive impairment and more pronounced in patients with AD (56–61). Majorly disturbed metabolic pathways observed in PD are also related to the metabolism of lipids, energy (TCA cycle, glycolysis, acylcarnitines), fatty acids and tryptophan, with the latter presenting a high correlation with the progression of PD (62–68). The energy and phospholipid metabolism have also been found to be impaired in patients with HD that ultimately affects the function of neurons (53, 69). Glucose metabolism is dysregulated in AD patients (70) and in area of the pons and cerebellum of MSA patients (71, 72), while an association of fatty acid metabolism with the development of ALS was observed (73). Finally, in our recent study we reported on the identification of metabolic biomarkers of early diagnosis and progression of FXTAS and on their association with altered lipid metabolism including free fatty acids, acylcarnitine, sphingolipids, diacylglycerol, and phospholipids, in individuals who developed the symptoms of FXTAS over time (25).

In this study we observed an association of metabolic biomarkers, including ceramides and mannonate, in CON as compared to NCON (**Figure 2**) with brain measures, specifically with area of the pons area, suggesting the potential role of altered metabolomics in the pathogenesis of FXTAS. We also found their significant association with the FXTAS stage (**Table 2**) ultimately providing the insight into the FXTAS disease progression with the dysregulation of the metabolic pathways.

The Krebs cycle or the TCA cycle is an important pathway in the production of ATP through the oxidative phosphorylation of acetyl-CoA in the mitochondria. With the onset of the neurodegenerative processes in PD, the metabolism of TCA cycle

TABLE 2 | Metabolite expression correlated with progression of FXTAS.

Sr #	Super pathway	Metabolite	Regression slope (95% CI)	Raw *P*-value	Adjusted *P*-value
1	Lipid	Myristate (14:0)	0.805 (0.376, 1.233)	< 0.001	0.0541
2	Lipid	Pentadecanoate (15:0)	1.4 (0.569, 2.241)	0.00239	0.0541
3	Lipid	1-oleoylglycerol (18:1)	1.02 (0.399, 1.639)	0.00284	0.0541
4	Lipid	Margarate (17:0)	1.1 (0.429, 1.766)	0.00287	0.0541
5	Lipid	(14 or 15)-methylpalmitate (a17:0 or i17:0)	0.89 (0.347, 1.432)	0.00288	0.0541
6	Lipid	(2 or 3)-decenoate (10:1n7 or n8)	0.763 (0.273, 1.253)	0.00426	0.058
7	Lipid	Palmitate (16:0)	0.86 (0.30, 1.42)	0.00469	0.058
8	Lipid	10-heptadecenoate (17:1n7)	0.515 (0.175, 0.855)	0.00516	0.058
9	Xenobiotics	Mannonate*	1.87 (0.62, 3.12)	0.00566	0.058
10	Lipid	Behenate (22:0)*	0.817 (0.258, 1.376)	0.0066	0.058
11	Amino Acid	Trans-urocanate	−1.32 (−2.227, −0.404)	0.00716	0.058
12	Amino Acid	8-methoxykynurenate	1.36 (0.402, 2.313)	0.00796	0.058
13	Lipid	Ceramide (d16:1/24:1, d18:1/22:1)*	0.795 (0.232, 1.359)	0.00828	0.058
14	Xenobiotics	7-methylurate	−0.633 (−1.085, −0.181)	0.00868	0.058
15	Xenobiotics	3-bromo-5-chloro-2,6-dihydroxybenzoic acid*	0.847 (0.228, 1.467)	0.01013	0.058
16	Lipid	10-undecenoate (11:1n1)	0.995 (0.265, 1.725)	0.01035	0.058
17	Lipid	Ceramide (d18:1/17:0, d17:1/18:0)*	1.34 (0.35, 2.32)	0.01075	0.058
18	Lipid	Myristoleate (14:1n5)	0.392 (0.100, 0.684)	0.01127	0.058
19	Lipid	Palmitoylcarnitine (C16)	1.9 (0.469, 3.335)	0.01212	0.058
20	Lipid	Lactosyl-N-behenoyl-sphingosine (d18:1/22:0)*	−2.56 (−4.487, −0.624)	0.01235	0.058
21	Lipid	5-dodecenoate (12:1n7)	0.618 (0.139, 1.097)	0.0143	0.0595
22	Lipid	Palmitoleate (16:1n7)	0.323 (0.0722, 0.5745)	0.01451	0.0595
23	Lipid	N-behenoyl-sphingadienine (d18:2/22:0)*	1.69 (0.365, 3.010)	0.01525	0.0595
24	Lipid	Arachidate (20:0)	3.32 (0.71, 5.93)	0.01554	0.0595
25	Xenobiotics	3,7-dimethylurate	−0.652 (−1.167, −0.138)	0.01583	0.0595
26	Energy	Fumarate	2.13 (0.435, 3.828)	0.01663	0.0598
27	Lipid	3-hydroxymyristate	0.701 (0.14, 1.26)	0.01719	0.0598

**The identity of these metabolites has not been officially confirmed based on the standard.*

was found to be dysregulated indicating an energy shortage and mitochondrial dysfunction in PD (74). Similarly, previous studies in FXTAS (19, 20, 22) reported on altered plasma and PBMCs levels (either increased or decreased) of several intermediates of the Krebs cycle in individuals with FXTAS as compared to controls. In accordance with these previous studies, we found a significance correlation of various Krebs cycle intermediates, including palmitate (16:0), palmitoleate (16:1n7), palmitoylcarnitine (C16) and fumarate (**Figure 3B**, bold) with the FXTAS stage (**Figure 3A**) supporting the observed mitochondrial dysfunction as a contributing factor in the pathogenesis of the FXTAS.

Sphingolipids include ceramides, sphingosine-1-phosphate, lactosyl-N-behenoyl-sphingosine and sphingomyelins, which play an important role in neuronal functions as sphingolipids are critical to prevent the cell death, loss of synaptic plasticity, and neurodegeneration (75). High levels of ceramide have been detected in the CNS and in plasma of AD patients and of PD patients, indicating that ceramide metabolism could be associated with various stages of PD and AD progression and hippocampal atrophy (76–78) and suggested as a pharmacological target for the AD treatment (79). In a recent study, the sphingolipid metabolism, and specifically the levels of sphingosine, sphingosine 1-phosphate, and sphingomyelin were found to be altered in the cerebellum of FXTAS mice (24). We have reported on increased ceramides levels in the CON as compared to NCON group (25) and, interestingly, in this study we observed a significant association with area of the pons both at V1 and V2 (**Figures 2A,B**). Further, the sphingolipid metabolism intermediates lactosyl-N-behenoyl-sphingosine (d18:1/22:0) and ceramide (d16:1/24:1, d18:1/22:1) (**Figure 3C**, bold) both were significantly associated with FXTAS stage suggesting their role in the development of FXTAS and the pathway as a potential target for personalized therapeutic development.

CONCLUSION

In this study, we found a significant correlation of metabolic biomarkers with the area of the pons in individuals who developed FXTAS over the time. We also report their significant association with the progression of the disorder and their role in context of dysregulated lipid and sphingolipid metabolism. These findings could be of a great value as the area of the pons provides distinct information about neuroanatomical

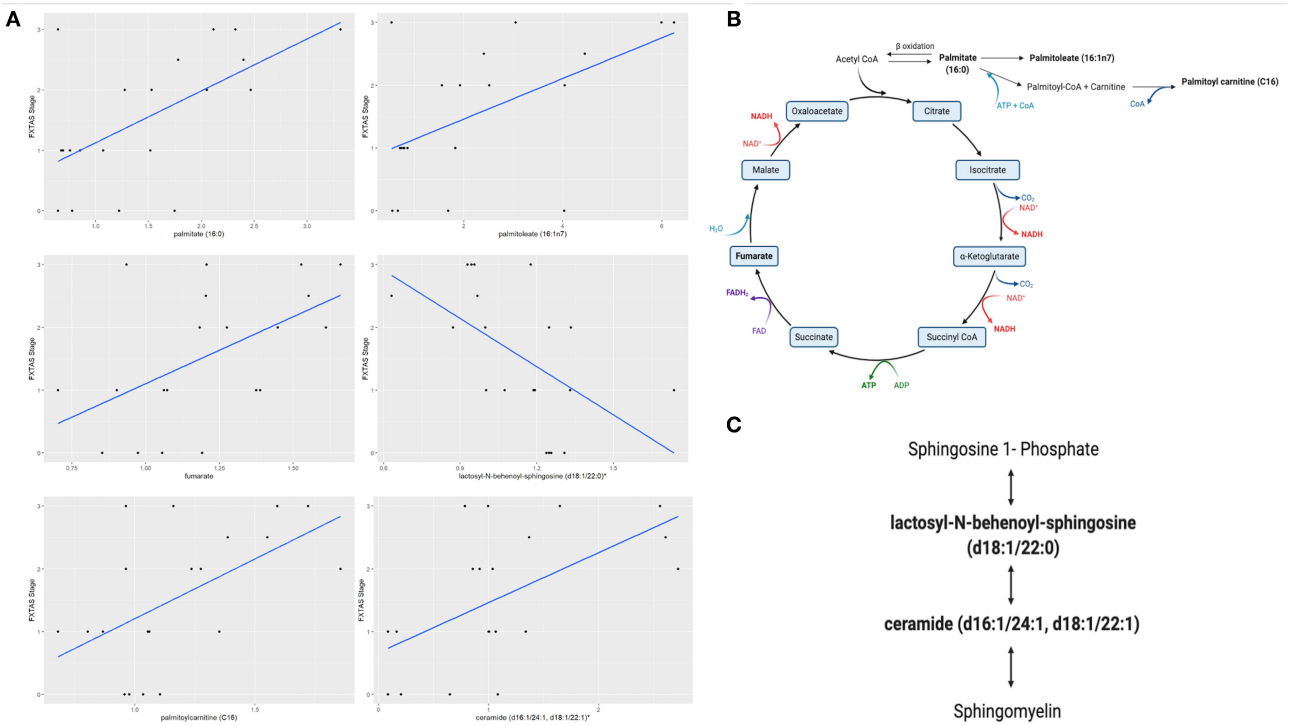

FIGURE 3 | Fatty acid and sphingolipids metabolism associated with FXTAS development. **(A)** Six identified metabolites in FXTAS associated with other neurodegenerative disorder, here the comparison with the FXTAS stage is shown; data are presented as FXTAS stage (*y*-axis) and associated metabolite (*x*-axis) by using scatter plots. **(B)**. Disturbance of fatty acid and lipids metabolism pathway is shown. **(C)** Sphingolipid metabolism pathway is shown. Bold metabolites, $p \leq 0.05$ linked with the development of FXTAS. *The identity of these metabolites has not been officially confirmed based on the standard.

and pathophysiological processes. Its association with the FXTAS biomarkers can assist in identifying the PM at risk as well as assist in evaluating disease progression and therapeutic responses to targeted drug development. Further research is needed to replicate these findings in a larger well-characterized cohort to further explore the role of other brainstem structures in FXTAS and human health and disease.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

This study and all the included protocols involved the human participant and were carried out in accordance with the Institutional Review Board at the University of California Davis. All participants gave written informed consent before participating in the study in line with the Declaration of Helsinki.

AUTHOR CONTRIBUTIONS

FT contributed to the conception while MZ, SR, DH, and FT contributed to the organization of the study. BD-J performed the statistical analysis and MZ wrote the first draft of the manuscript. MZ, BD-J, EF, SR, DH, and FT made amendments to the manuscripts. All authors contributed to the manuscript revision and approved the submitted version, recruited samples in their respective centers, assessed outpatients, and organized the database.

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REFERENCES

1. Niccoli T, Partridge L. Ageing as a risk factor for disease. *Curr Biol.* (2012) 22:R741–52. doi: 10.1016/j.cub.2012.07.024
2. Schönfeld P, Reiser G. Why does brain metabolism not favor burning of fatty acids to provide energy? Reflections on disadvantages of the use of free fatty acids as fuel for brain. *J Cereb Blood Flow Metab.* (2013) 33:1493–9. doi: 10.1038/jcbfm.2013.128
3. Camandola S, Mattson MP. Brain metabolism in health, aging, and neurodegeneration. *EMBO J.* (2017) 36:1474–92. doi: 10.15252/embj.201695810
4. Hagerman RJ, Berry-Kravis E, Hazlett HC, Bailey DB Jr, Moine H, Kooy RF, et al. Fragile X syndrome. *Nat Rev Dis Primers.* (2017) 3:17065. doi: 10.1038/nrdp.2017.65
5. Jacquemont S, Hagerman RJ, Leehey MA, Hall DA, Levine RA, Brunberg JA, et al. Penetrance of the fragile X-associated tremor/ataxia syndrome in a premutation carrier population. *JAMA.* (2004) 291:460–9. doi: 10.1001/jama.291.4.460
6. Tassone F, Iong KP, Tong T-H, Lo J, Gane LW, Berry-Kravis E, et al. *FMR1* CGG allele size and prevalence ascertained through newborn screening in the United States. *Genome Med.* (2012) 4:100. doi: 10.1186/gm401
7. Hagerman RJ, Hagerman P. Fragile X-associated tremor/ataxia syndrome - features, mechanisms and management. *Nat Rev Neurol.* (2016) 12:403–412. doi: 10.1038/nrneurol.2016.82
8. Rivera SM, Stebbins GT, Grigsby J. Radiological Findings in FXTAS. In: *The Fragile X-Associated Tremor Ataxia Syndrome (FXTAS)* New York, NY: Springer. p. 55–66.
9. Apartis E, Blancher A, Meissner WG, Guyant-Maréchal L, Maltête D, De Broucker T, et al. FXTAS: new insights and the need for revised diagnostic criteria. *Neurology.* (2012) 79:1898–907. doi: 10.1212/WNL.0b013e318271f7ff
10. Kalus S, King J, Lui E, Gaillard F. Fragile X-associated tremor/ataxia syndrome: an under-recognised cause of tremor and ataxia. *J Clin Neurosci.* (2016) 23:162–4. doi: 10.1016/j.jocn.2015.08.010
11. Scaglione C, Ginestroni A, Vella A, Dotti MT, Nave RD, Rizzo G, et al. MRI and SPECT of midbrain and striatal degeneration in fragile X-associated tremor/ataxia syndrome. *J Neurol.* (2008) 255:144–6. doi: 10.1007/s00415-007-0711-8
12. Grinberg LT, Rueb U, Heinsen H. Brainstem: neglected locus in neurodegenerative diseases. *Front Neurol.* (2011) 2:42. doi: 10.3389/fneur.2011.00042
13. Yang A, h. Xiao X, l. Wang Z. Evaluation of normal changes in pons metabolites due to aging using turbo spectroscopic imaging. *AJNR Am J Neuroradiol.* (2014) 35:2099–105. doi: 10.3174/ajnr.A4031
14. Emamzadeh FN, Surguchov A. Parkinson's disease: biomarkers, treatment, and risk factors. *Front Neurosci.* (2018) 12:612. doi: 10.3389/fnins.2018.00612
15. Chelban V, Bocchetta M, Hassanein S, Haridy NA, Houlden H, Rohrer JD. An update on advances in magnetic resonance imaging of multiple system atrophy. *J Neurol.* (2019) 266:1036–45. doi: 10.1007/s00415-018-9121-3
16. Shelton AL, Wang JY, Fourie E, Tassone F, Chen A, Frizzi L, et al. Middle cerebellar peduncle width-a novel mri biomarker for FXTAS? *Front Neurosci.* (2018) 12:379. doi: 10.3389/fnins.2018.00379
17. Zafarullah M, Tang H-T, Durbin-Johnson B, Fourie E, Hessl D, Rivera SM, et al. *FMR1* locus isoforms: potential biomarker candidates in fragile X-associated tremor/ataxia syndrome (FXTAS). *Sci Rep.* (2020) 10:11099. doi: 10.1038/s41598-020-67946-y
18. Cenini G, Lloret A, Cascella R. Oxidative stress in neurodegenerative diseases: from a mitochondrial point of view. *Oxid Med Cell Longev.* (2019) 2019:2105607. doi: 10.1155/2019/2105607
19. Giulivi C, Napoli E, Tassone F, Halmai J, Hagerman R. Plasma biomarkers for monitoring brain pathophysiology in *fmr1* premutation carriers. *Front Mol Neurosci.* (2016) 9:71. doi: 10.3389/fnmol.2016.00071
20. Giulivi C, Napoli E, Tassone F, Halmai J, Hagerman R. Plasma metabolic profile delineates roles for neurodegeneration, pro-inflammatory damage and mitochondrial dysfunction in the *FMR1* premutation. *Biochem J.* (2016) 473:3871–88. doi: 10.1042/BCJ20160585
21. Song G, Napoli E, Wong S, Hagerman R, Liu S, Tassone F, et al. Altered redox mitochondrial biology in the neurodegenerative disorder fragile X-tremor/ataxia syndrome: use of antioxidants in precision medicine. *Mol Med.* (2016) 22:548–59. doi: 10.2119/molmed.2016.00122
22. Napoli E, Song G, Schneider A, Hagerman R, Eldeeb MAAA, Azarang A, et al. Warburg effect linked to cognitive-executive deficits in *FMR1* premutation. *FASEB J.* (2016) 30:3334–51. doi: 10.1096/fj.201600315R
23. Napoli E, Schneider A, Wang JY, Trivedi A, Carrillo NR, Tassone F, et al. Allopregnanolone treatment improves plasma metabolomic profile associated with GABA metabolism in fragile X-associated tremor/ataxia syndrome: a pilot study. *Mol Neurobiol.* (2019) 56:3702–13. doi: 10.1007/s12035-018-1330-3
24. Kong HE, Lim J, Zhang F, Huang L, Gu Y, Nelson DL, et al. Metabolic pathways modulate the neuronal toxicity associated with fragile X-associated tremor/ataxia syndrome. *Hum Mol Genet.* (2019) 28:980–91. doi: 10.1093/hmg/ddy410
25. Zafarullah M, Palczewski G, Rivera SM, Hessl DR, Tassone F. Metabolic profiling reveals dysregulated lipid metabolism and potential biomarkers associated with the development and progression of Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS). *FASEB J.* (2020) 34:16676–92. doi: 10.1096/fj.202001880R
26. Bacalman S, Farzin F, Bourgeois JA, Cogswell J, Goodlin-Jones BL, Gane LW, et al. Psychiatric phenotype of the fragile X-associated tremor/ataxia syndrome (FXTAS) in males: newly described fronto-subcortical dementia. *J Clin Psychiatry.* (2006) 67:87–94. doi: 10.4088/JCP.v67n0112
27. Zafarullah M, Tassone F. Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS). *Methods Mol Biol.* (2019) 1942:173–89. doi: 10.1007/978-1-4939-9080-1_15
28. Tassone F, Pan R, Amiri K, Taylor AK, Hagerman PJ. A rapid polymerase chain reaction-based screening method for identification of all expanded alleles of the fragile X (*FMR1*) gene in newborn and high-risk populations. *J Mol Diagn.* (2008) 10:43–9. doi: 10.2353/jmoldx.2008.070073
29. Filipovic-Sadic S, Sah S, Chen L, Krosting J, Sekinger E, Zhang W, et al. A novel *FMR1* PCR method for the routine detection of low abundance expanded alleles and full mutations in fragile X syndrome. *Clin Chem.* (2010) 56:399–408. doi: 10.1373/clinchem.2009.136101
30. Ardekani BA, Bachman AH. Model-based automatic detection of the anterior and posterior commissures on MRI scans. *Neuroimage.* (2009) 46:677–82. doi: 10.1016/j.neuroimage.2009.02.030
31. Mori S, Crain BJ, Chacko VP, van Zijl PC. Three-dimensional tracking of axonal projections in the brain by magnetic resonance imaging. *Ann Neurol.* (1999) 45:265–9.
32. Tustison NJ, Avants BB, Cook PA, Zheng Y, Egan A, Yushkevich PA, et al. N4ITK: improved N3 bias correction. *IEEE Trans Med Imaging.* (2010) 29:1310–20. doi: 10.1109/TMI.2010.2046908
33. Quattrone A, Nicoletti G, Messina D, Fera F, Condino F, Pugliese P, et al. MR imaging index for differentiation of progressive supranuclear palsy from Parkinson disease and the Parkinson variant of multiple system atrophy. *Radiology.* (2008) 246:214–21. doi: 10.1148/radiol.2453061703
34. Nicoletti G, Fera F, Condino F, Auteri W, Gallo O, Pugliese P, et al. MR imaging of middle cerebellar peduncle width: differentiation of multiple system atrophy from Parkinson disease. *Radiology.* (2006) 239:825–30. doi: 10.1148/radiol.2393050459
35. Evans AM, DeHaven CD, Barrett T, Mitchell M, Milgram E. Integrated, nontargeted ultrahigh performance liquid chromatography/electrospray ionization tandem mass spectrometry platform for the identification and relative quantification of the small-molecule complement of biological systems. *Anal Chem.* (2009) 81:6656–67. doi: 10.1021/ac901536h
36. Benjamini Y, Hochberg, Y. Controlling the false discovery rate: a practical and powerful approach to multiple testing. *J R Stat Soc Ser.* (1995) 57:289–300. doi: 10.1111/j.2517-6161.1995.tb02031.x
37. The R Project for Statistical Computing. Available online at: https://www.r-project.org/ (accessed April 6, 2021)
38. Saeed U, Compagnone J, Aviv RI, Strafella AP, Black SE, Lang AE, et al. Imaging biomarkers in Parkinson's disease and Parkinsonian syndromes: current and emerging concepts. *Transl Neurodegener.* (2017) 6:8. doi: 10.1186/s40035-017-0076-6

39. Mazón M, Vázquez Costa JF, Ten-Esteve A, Martí-Bonmatí L. Imaging biomarkers for the diagnosis and prognosis of neurodegenerative diseases. the example of amyotrophic lateral sclerosis. *Front Neurosci.* (2018) 12:784. doi: 10.3389/fnins.2018.00784
40. Braun DJ, Van Eldik LJ. In vivo brainstem imaging in alzheimer's disease: potential for biomarker development. *Front Aging Neurosci.* (2018) 10:266. doi: 10.3389/fnagi.2018.00266
41. Kehoe EG, McNulty JP, Mullins PG, Bokde AL. Advances in MRI biomarkers for the diagnosis of Alzheimer's disease. *Biomark Med.* (2014) 8:1151–69. doi: 10.2217/bmm.14.42
42. Del Negro CA, Funk GD, Feldman JL. Breathing matters. *Nat Rev Neurosci.* (2018) 19:351–67. doi: 10.1038/s41583-018-0003-6
43. Sara SJ. The locus coeruleus and noradrenergic modulation of cognition. *Nat Rev Neurosci.* (2009) 10:211–23. doi: 10.1038/nrn2573
44. Przedborski S. The two-century journey of Parkinson disease research. *Nat Rev Neurosci.* (2017) 18:251–9. doi: 10.1038/nrn.2017.25
45. Elvsåshagen T, Bahrami S, van der Meer D, Agartz I, Alnæs D, Barch DM, et al. The genetic architecture of human brainstem structures and their involvement in common brain disorders. *Nat Commun.* (2020) 11:4016. doi: 10.1038/s41467-020-17376-1
46. Reginold W, Lang AE, Marras C, Heyn C, Alharbi M, Mikulis DJ. Longitudinal quantitative MRI in multiple system atrophy and progressive supranuclear palsy. *Parkinsonism Relat Disord.* (2014) 20:222–5. doi: 10.1016/j.parkreldis.2013.10.002
47. Ito M, Watanabe H, Kawai Y, Atsuta N, Tanaka F, Naganawa S, et al. Usefulness of combined fractional anisotropy and apparent diffusion coefficient values for detection of involvement in multiple system atrophy. *J Neurol Neurosurg Psychiatry.* (2007) 78:722–8. doi: 10.1136/jnnp.2006.104075
48. Boxer AL, Geschwind MD, Belfor N, Gorno-Tempini ML, Schauer GF, Miller BL, et al. Patterns of brain atrophy that differentiate corticobasal degeneration syndrome from progressive supranuclear palsy. *Arch Neurol.* (2006) 63:81–6. doi: 10.1001/archneur.63.1.81
49. Theofilas P, Ehrenberg AJ, Dunlop S, Di Lorenzo Alho AT, Nguy A, Leite REP, et al. Locus coeruleus volume and cell population changes during Alzheimer's disease progression: A stereological study in human postmortem brains with potential implication for early-stage biomarker discovery. *Alzheimers Dement.* (2017) 13:236–46. doi: 10.1016/j.jalz.2016.06.2362
50. Bourgognon J-M, Steinert JR. The metabolome identity: basis for discovery of biomarkers in neurodegeneration. *Neural Regen Res.* (2019) 14:387–90. doi: 10.4103/1673-5374.245464
51. Wilkins JM, Trushina E. Application of metabolomics in Alzheimer's disease. *Front Neurol.* (2018) 8:719. doi: 10.3389/fneur.2017.00719
52. Shao Y, Le W. Recent advances and perspectives of metabolomics-based investigations in Parkinson's disease. *Mol Neurodegener.* (2019) 14:3. doi: 10.1186/s13024-018-0304-2
53. Graham SF, Pan X, Yilmaz A, Macias S, Robinson A, Mann D, et al. Targeted biochemical profiling of brain from Huntington's disease patients reveals novel metabolic pathways of interest. *Biochim Biophys Acta Mol Basis Dis.* (2018) 1864:2430–7. doi: 10.1016/j.bbadis.2018.04.012
54. Stoessel D, Stellmann J-P, Willing A, Behrens B, Rosenkranz SC, Hodecker SC, et al. Metabolomic profiles for primary progressive multiple sclerosis stratification and disease course monitoring. *Front Hum Neurosci.* (2018) 12:226. doi: 10.3389/fnhum.2018.00226
55. Kirk SE, Tracey TJ, Steyn FJ, Ngo ST. Biomarkers of metabolism in amyotrophic lateral sclerosis. *Front Neurol.* (2019) 10:191. doi: 10.3389/fneur.2019.00191
56. Trushina E, Dutta T, Persson XM, Mielke MM, Petersen RC. Identification of altered metabolic pathways in plasma and CSF in mild cognitive impairment and Alzheimer's disease using metabolomics. *PLoS ONE.* (2013) 8:e63644. doi: 10.1371/journal.pone.0063644
57. Kaddurah-Daouk R, Zhu H, Sharma S, Bogdanov M, Rozen SG, Matson W, et al. Alterations in metabolic pathways and networks in Alzheimer's disease. *Transl Psychiatry.* (2013) 3:e244. doi: 10.1038/tp.2013.18
58. Graham SF, Chevallier OP, Elliott CT, Hölscher C, Johnston J, McGuinness B, et al. Untargeted metabolomic analysis of human plasma indicates differentially affected polyamine and L-arginine metabolism in mild cognitive impairment subjects converting to Alzheimer's disease. *PLoS ONE.* (2015) 10:e0119452. doi: 10.1371/journal.pone.0119452
59. Sonntag K-C, Ryu W-I, Amirault KM, Healy RA, Siegel AJ, McPhie DL, et al. Late-onset Alzheimer's disease is associated with inherent changes in bioenergetics profiles. *Sci Rep.* (2017) 7:14038. doi: 10.1038/s41598-017-14420-x
60. Mapstone M, Cheema AK, Fiandaca MS, Zhong X, Mhyre TR, MacArthur LH, et al. Plasma phospholipids identify antecedent memory impairment in older adults. *Nat Med.* (2014) 20:415–8. doi: 10.1038/nm.3466
61. Toledo JB, Arnold M, Kastenmüller G, Chang R, Baillie RA, Han X, et al. Metabolic network failures in Alzheimer's disease: a biochemical road map. *Alzheimers Dement.* (2017) 13:965–84. doi: 10.1016/j.jalz.2017.01.020
62. Roede JR, Uppal K, Park Y, Lee K, Tran V, Walker D, et al. Serum metabolomics of slow vs. rapid motor progression Parkinson's disease: a pilot study. *PLoS ONE.* (2013) 8:e77629. doi: 10.1371/journal.pone.0077629
63. Trupp M, Jonsson P, Ohrfelt A, Zetterberg H, Obudulu O, Malm L, et al. Metabolite and peptide levels in plasma and CSF differentiating healthy controls from patients with newly diagnosed Parkinson's disease. *J Parkinsons Dis.* (2014) 4:549–60. doi: 10.3233/JPD-140389
64. Lei S, Zavala-Flores L, Garcia-Garcia A, Nandakumar R, Huang Y, Madayiputhiya N, et al. Alterations in energy/redox metabolism induced by mitochondrial and environmental toxins: a specific role for glucose-6-phosphate-dehydrogenase and the pentose phosphate pathway in paraquat toxicity. *ACS Chem Biol.* (2014) 9:2032–48. doi: 10.1021/cb400894a
65. Schulte EC, Altmaier E, Berger HS, Do KT, Kastenmüller G, Wahl S, et al. Alterations in lipid and inositol metabolisms in two dopaminergic disorders. *PLoS ONE.* (2016) 11:e0147129. doi: 10.1371/journal.pone.0147129
66. Saiki S, Hatano T, Fujimaki M, Ishikawa K-I, Mori A, Oji Y, et al. Decreased long-chain acylcarnitines from insufficient β-oxidation as potential early diagnostic markers for Parkinson's disease. *Sci Rep.* (2017) 7:7328. doi: 10.1038/s41598-017-06767-y
67. Havelund JF, Andersen AD, Binzer M, Blaabjerg M, Heegaard NHH, Stenager E, et al. Changes in kynurenine pathway metabolism in Parkinson patients with L-DOPA-induced dyskinesia. *J Neurochem.* (2017) 142:756–66. doi: 10.1111/jnc.14104
68. Okuzumi A, Hatano T, Ueno S-I, Ogawa T, Saiki S, Mori A, et al. Metabolomics-based identification of metabolic alterations in PARK2. *Ann Clin Transl Neurol.* (2019) 6:525–36. doi: 10.1002/acn3.724
69. Kumar KK, Goodwin CR, Uhouse MA, Bornhorst J, Schwerdtle T, Aschner M, et al. Untargeted metabolic profiling identifies interactions between Huntington's disease and neuronal manganese status. *Metallomics.* (2015) 7:363–70. doi: 10.1039/C4MT00223G
70. An Y, Varma VR, Varma S, Casanova R, Dammer E, Pletnikova O, et al. Evidence for brain glucose dysregulation in Alzheimer's disease. *Alzheimers Dement.* (2018) 14:318–29. doi: 10.1016/j.jalz.2017.09.011
71. Juh R, Kim J, Moon D, Choe B, Suh T. Different metabolic patterns analysis of Parkinsonism on the 18F-FDG PET. *Eur J Radiol.* (2004) 51:223–33. doi: 10.1016/S0720-048X(03)00214-6
72. Eckert T, Barnes A, Dhawan V, Frucht S, Gordon MF, Feigin AS, et al. FDG PET in the differential diagnosis of parkinsonian disorders. *Neuroimage.* (2005) 26:912–21. doi: 10.1016/j.neuroimage.2005.03.012
73. Goutman SA, Boss J, Guo K, Alakwaa FM, Patterson A, Kim S, et al. Untargeted metabolomics yields insight into ALS disease mechanisms. *J Neurol Neurosurg Psychiatry.* (2020) 91:1329–38. doi: 10.1136/jnnp-2020-323611
74. Graham SF, Rey NL, Yilmaz A, Kumar P, Madaj Z, Maddens M, et al. Biochemical profiling of the brain and blood metabolome in a mouse model of prodromal Parkinson's disease reveals distinct metabolic profiles. *J Proteome Res.* (2018) 17:2460–9. doi: 10.1021/acs.jproteome.8b00224
75. Alessenko AV, Albi E. Exploring sphingolipid implications in neurodegeneration. *Front Neurol.* (2020) 11:437. doi: 10.3389/fneur.2020.00437
76. Di Pardo A, Maglione V. Sphingolipid metabolism: a new therapeutic opportunity for brain degenerative disorders. *Front Neurosci.* (2018) 12:249. doi: 10.3389/fnins.2018.00249
77. Kim M, Nevado-Holgado A, Whiley L, Snowden SG, Soininen H, Kloszewska I, et al. Association between plasma ceramides and phosphatidylcholines and hippocampal brain volume in late onset

Alzheimer's disease. *J Alzheimers Dis.* (2017) 60:809–17. doi: 10.3233/JAD-160645

78. Mielke MM, Maetzler W, Haughey NJ, Bandaru VVR, Savica R, Deuschle C, et al. Plasma ceramide and glucosylceramide metabolism is altered in sporadic Parkinson's disease and associated with cognitive impairment: a pilot study. *PLoS ONE.* (2013) 8:e73094. doi: 10.1371/journal.pone.0073094
79. van Kruining D, Luo Q, van Echten-Deckert G, Mielke MM, Bowman A, Ellis S, et al. Sphingolipids as prognostic biomarkers of neurodegeneration, neuroinflammation, and psychiatric diseases and their emerging role in lipidomic investigation methods. *Adv Drug Deliv Rev.* (2020) 159:232–44. doi: 10.1016/j.addr.2020.04.009

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Do Fragile X Syndrome and Other Intellectual Disorders Converge at Aberrant Pre-mRNA Splicing?

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Fragile X Syndrome is a neuro-developmental disorder caused by the silencing of the FMR1 gene, resulting in the loss of its protein product, FMRP. FMRP binds mRNA and represses general translation in the brain. Transcriptome analysis of the Fmr1-deficient mouse hippocampus reveals widespread dysregulation of alternative splicing of pre-mRNAs. Many of these aberrant splicing changes coincide with those found in post-mortem brain tissue from individuals with autism spectrum disorders (ASDs) as well as in mouse models of intellectual disability such as PTEN hamartoma syndrome (PHTS) and Rett Syndrome (RTT). These splicing changes could result from chromatin modifications (e.g., in FXS, RTT) and/or splicing factor alterations (e.g., PTEN, autism). Based on the identities of the RNAs that are mis-spliced in these disorders, it may be that they are at least partly responsible for some shared pathophysiological conditions. The convergence of splicing aberrations among these autism spectrum disorders might be crucial to understanding their underlying cognitive impairments.

Keywords: Fragile X syndrome, intellectual disability, autism, alternative splicing, gene regulation

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INTRODUCTION

Neurodevelopmental disorders (NDDs) including those that lie on the autism spectrum are diagnosed primarily based on behavioral presentations in early childhood. NDDs vary in severity of clinical presentations and most commonly occur as intellectual disability (ID) (1). Children with an autism spectrum disorder (ASD) often present with social and cognitive deficits, repetitive behaviors, language impairments, and intellectual disability that may range from mild to severe manifestations. The genetic heterogeneity and wide range of clinical presentations of ASD have hindered therapeutic advances. In 5–10% of ASD cases, the underlying causes are either single-gene mutations, chromosomal abnormalities, or environmental toxin exposure. Amongst the single-gene disorders, Fragile X Syndrome (FXS, caused by a triplet repeat expansion mutation in the FMR1 gene and subsequent loss of its protein product, FMRP) and the Rett syndrome (RTT, mutations in the MECP2 gene resulting in loss of MECP2 protein identified in 95% of the cases) are the most prevalent. Indeed 5% of ASD cases harbor the FXS mutation, and 50% of FXS patients are on the autism spectrum. About 50% of RTT patients are initially diagnosed with autism during the active regression period in Stage 2; however, upon progression to Stage 3; autistic features persist in only 19% of RTT patients (2). Understanding single gene intellectual disabilities (IDs) might aid in a better understanding of ASD, which currently has a prevalence of 1 in every 54 children aged 8 years in the US with co-diagnosis of ID in 33% of the children (3). Neurodevelopmental delays along with cognitive impairments are thought to stem from synaptic structure and function aberrations that are characteristic of ASDs and monogenic IDs such as FXS,

RTT, tuberous sclerosis (TSC caused by mutations in the TSC1/TSC2 complex). Molecular analysis of patient-derived tissues and mouse models of the monogenic IDs has shown widespread changes at the epigenetic, transcriptional, and translational gene expression levels. The interplay between changes at multiple levels of gene regulation might be essential to the pathophysiology of a disorder. For example, in FXS, loss of FMRP protein resulting in increase in protein synthesis of FMRP target RNAs along with changes in multiple molecular pathways have been identified [reviewed in Ref. (4)]. Detailed molecular analysis of targets of translational de-repression in FXS led to the finding that a chromatin modifier, SETD2 (SET domain containing methyltransferase protein), is altered, resulting in downstream alterations in the chromatin landscape and genome-wide aberrant alternative splicing of mRNAs (5). Aberrant alternative splicing has previously been studied in complex NDDs such as ASD (6–8) and other psychiatric illnesses with a neurodevelopmental trajectory including Schizophrenia (8, 9), Bipolar Disorder (8, 9), Huntington's (10), and recently in monogenic IDs such as FXS (5), PTEN (11), and RTT (12–14) (**Table 1**).

Alternative pre-mRNA splicing generates multiple transcript isoforms and protein variants for a single gene by co-transcriptionally altering the exon composition of the mature mRNA (**Figure 1**). Alternatively spliced transcripts are widely prevalent in human cells and tissues, resulting in multiple transcript isoforms for 95% of the multiexon genes, generating a ∼10-fold increase in the number of transcript isoforms per gene. With the advent of high-throughput transcriptomics, the alternative splicing landscape of cells and tissues can be readily investigated. Alternative splicing is detected in all metazoans and is closely correlated with organismal complexity (25). Furthermore, alternative splicing patterns are tissue- and cell-type-specific (10, 26), and in vertebrates, mainly contribute to the development and function of the central nervous system. Some neuronal genes such as Neurexins, n-Cadherins, and calcium-activated potassium channels can produce hundreds of mRNA isoforms resulting in a functionally diverse protein arsenal for efficient neuron functioning. Indeed, pathological consequences such as neurodegenerative, neurodevelopmental, and complex disorders including ASD occur when alternative splicing goes awry. Extensive studies using post-mortem patient brain tissue transcriptomics from ASD patients have shown pervasive mis-regulation of microexon (exon size of 3–27 nt) splicing (6, 7, 15, 27, 28).

Using genome-wide screens to identify RNA-binding proteins that might be key to the splicing dysregulation, splicing factors such as SRRM4/nSR100 were identified. A significant proportion of ASD cases also harbor *de-novo* genetic mutations resulting in cryptic splice sites or mutations in RNA binding proteins [e.g., *RBFOX1* (29)] that regulate alternative splicing. Deep neural networks such as SpliceAI can predict deleterious genomic mutations in complex disorders such as ASD (30). Studies from our lab on FXS and others' on RTT, PTEN have also identified alternative splicing aberrations in genes with essential neuronal functions, suggesting that improper transcript isoform ratios may be a key feature of several IDs. Given the recent increase in studies identifying alternative splicing defects, it might be useful to encourage parallel assessment of IDs to help focus on functionally relevant changes. Indeed, the biological pathways that local splicing defects might disrupt are similar amongst mouse models of multiple monogenic IDs and tissues derived from patients with complex disorders like ASD (for example, neuron development, synaptic vesicle function, cytoskeleton formation). They may thus underlie the shared pathophysiology of IDs.

A recent review has emphasized the ubiquitin system (UbS) as a potential convergent pathway among IDs such as Angelman Syndrome, FXS, PHTS, Loeys-Dietz Syndrome (LDS), and ASD (31). Furthermore, several epigenetic factors are misregulated in these disorders, which might trigger long-lasting effects on the epigenetic landscape in IDs. Translational regulation might be another point of convergence as loss of FMRP in FXS results in an increase in the synthesis of proteins, at least in some cases, by alleviating ribosomes from being stalled on specific mRNAs. Similar mechanisms of translational deregulation have recently been reported in Huntington's disease mouse models. Although many of these pathways are perturbed amongst different monogenic IDs, the most striking genome-wide alterations in complex IDs such as ASD are aberrant alternative pre-mRNA splicing. Understanding the potential regulatory factors and points of convergence among different IDs might help early identification, monitor progression, and perhaps lead to common therapeutic advances. Thus, we will highlight emerging research focus on alternative splicing in various IDs affecting neurodevelopment and brain function.

ALTERNATIVE SPLICING DURING NEURODEVELOPMENT IN IDS

Precise temporal regulation of alternative splicing of pre-mRNAs plays an essential role in every step of neurodevelopment, including but not restricted to cortical development (32), cerebellar development (33), neuron subtype specificity (34, 35), neuronal maturation (36), and axon formation (37). Tightly regulated 'early switch' and 'late switch' exons are encoded by genes in which exons are spliced out around birth or post-natally during development in neurons isolated from the mouse cortex during development, reflecting progressive stages of neuronal maturation (38). These switches may be orchestrated by an interplay of cell type-specific expression of various splicing factors (36, 38–41). Using integrative approaches to assess splicing data from various types of neurons at different stages of maturation, a 'splicing code' has been proposed (38). Although the model currently uses only single exon changes, it does provide a foundation for future assessment of complex splicing patterns during neurodevelopment.

Another splicing program regulating mouse cerebellar cortex development and synapse maturation from P1 to P30 has been proposed to rely on the splicing factor SAM68 (33). Indeed, ablation of SAM68 resulted in impaired synaptic functions and long-lasting social interaction deficits in mouse models. Another splicing factor, nSR100/SRRM4, is crucial for regulating the alternative splicing program during

TABLE 1 | Evidence of differential alternative splicing in several neurodevelopmental disorders.

Disorder	Origin tissue	Species	Mechanism	References
Autism spectrum disorders	Frontal and temporal cerebral cortex	Homo sapiens	Regulation by splicing factors nSR100/Srrm4, RBFOX, and PTBP1 proteins	(6, 8, 15–17)
	Blood		–	(18)
Fragile X Syndrome	Hippocampal tissue slices	Mus musculus	Altered histone modifications at splice junctions of alternatively spliced exons (H3K36me3)	(5)
RTT	Hippocampus	Mus musculus	DNA modification 5hmC, and histone modifications (H3K4me3, H3K36me3)	(13, 19)
PTEN	Cortex	Mus musculus	Disruption of interactions with spliceosomal protein U2af2	(11)
Schizophrenia	Frontal and temporal cerebral cortex	Homo sapiens		(8, 9)
	Blood			(20)
Bipolar disorder	Frontal and temporal cerebral cortex	Homo sapiens		(8, 9)
	Blood			(20)
Huntington's disease	BA4 (Brodmann area 4) motor cortex	Homo sapiens	Regulated by splicing factor PTBP1	(21, 22)
		Drosophila melanogaster	Spliceosome proteins sequestered by mutant *Htt* mRNA.	(23)
Microcephaly	Fibroblast	Homo sapiens	Mutation in SNRPE gene results in failure to assemble the pre-mRNA processing complex U snRNPs.	(24)

Studies using various human patient tissues as well as mouse models of causal mutations for IDs show widespread presence of aberrant pre-mRNA splicing.

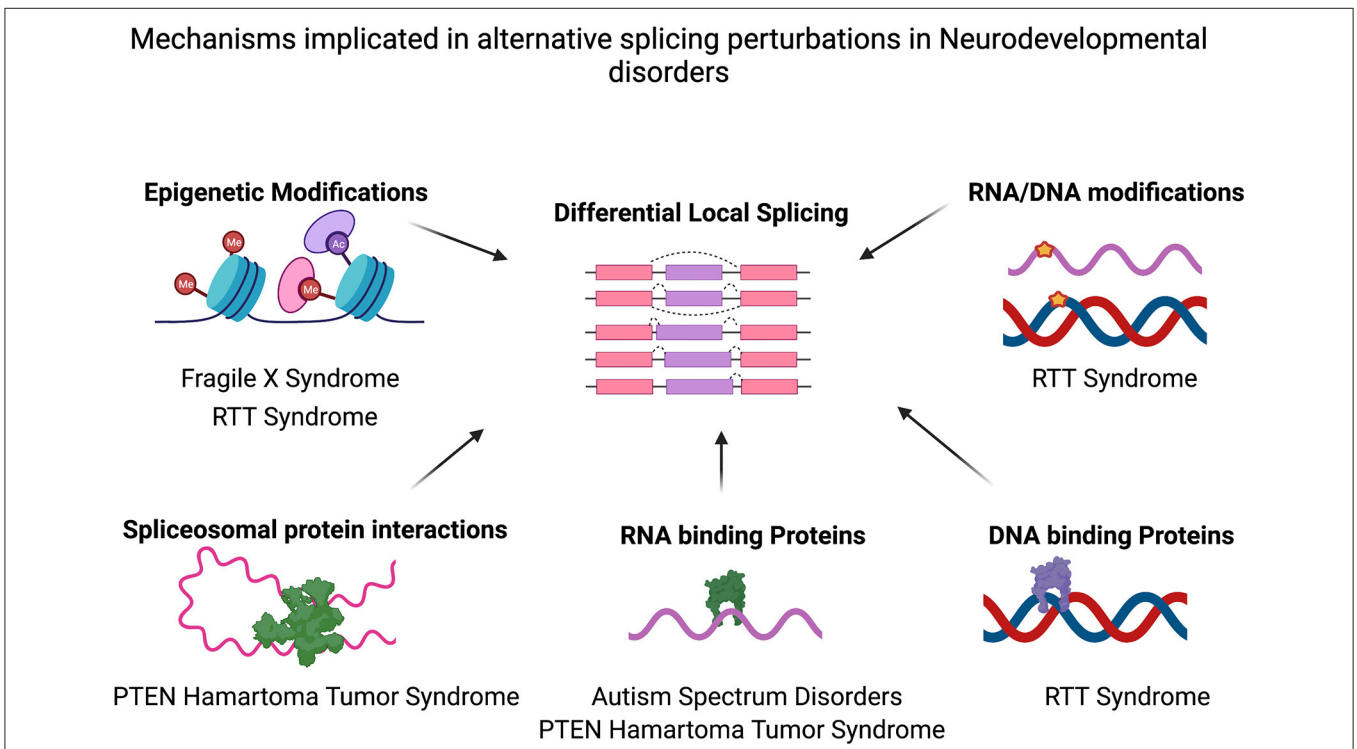

FIGURE 1 | Mechanisms implicated in alternative splicing perturbations in various neurodevelopmental disorders. Differential alternative splicing results in various transcript isoforms generated from a single gene via splicing events (for example, Exon Skipping, Mutually exclusive exon usage, Alternative 3′ Splice site/5′ splice site usage or intron retention). Various molecular mechanisms may cause the genome-wide alternative splicing perturbations identified in several neurodevelopmental disorders such as epigenetic modifications (e.g., in FXS, RTT Syndrome) and/or splicing factor alterations (e.g., PTEN, autism) or as a result of DNA modifications and differential binding of proteins to DNA (RTT Syndrome). Figure created with Biorender.com. Also see **Table 1**.

neurodevelopment, especially microexon (3–27 nt) splicing (7). Microexons represent a class of exons that are enriched in protein-protein interaction domains that are associated with cell signaling and, due to their small size, have the highest tendency to be included in neuronal transcripts (6). An hypothesis for differential splicing of microexons (defined as <51 nt in this study) is that various genomic signatures such as the presence of intronic enhancers that dictate binding of splicing regulators including PTBP1 and RBFOX or alterations of the thymine content of the microexons may dictate their splicing patterns (16). Microexon mis-splicing occurs extensively in ASD, identified via large-scale transcriptomic studies from post-mortem cortex tissue from patients (6, 7, 11, 42). For example, the *CPEB4* (Cytoplasmic polyadenylation element binding proteins 4) gene regulates translation of mRNAs by altering their poly(A)-tails and plays an important role in embryonic development and synaptic plasticity. In ASD patients, a neuron specific microexon of *CPEB4* (Exon 4) which contains post-translational modification sites was found to be skipped to a greater extent resulting in changes in translation of downstream *CPEB4* target genes. Indeed, using a mouse model with deletion of *CPEB4*- Exon 4 a potential functional role for the microexon skipping was assayed. Mutant mice displayed changes in polyadenylated transcriptome corresponding to changes in high-risk expression ASD genes (e.g., *PTEN, RBFOX1, AUTS2*) and mimicked ASD phenotypes such as deficits in spine density, reduced number of excitatory synapses along with typical ASD behaviors such as stereotypic running and diminished social interaction (28). However, an important caveat to this study is that the physiological change in the exon 4 of *CPEB4* in ASD patient brain tissue was a modest exon inclusion difference (percent spliced in (PSI) change, ASD vs. control) = −7.6%. Similar to ASD, the inclusion level difference of exons in various IDs for most genes is modest, and hence their impact on pathophysiology needs further investigation.

Alternative splicing programs are also constantly altered during aging (43–45), and their disruption can result in the early onset of neurodegeneration (43, 44, 46). Thus, evaluating age-specific disruptions in alternative splicing patterns could help assess developmental delays in IDs. For example, assessing the splicing patterns in PTEN mouse models at P14 (post-natal day 14) and P40 (post-natal day 40) time points highlighted age-specific mis-spliced isoforms that might disrupt distinct signaling pathways, partly due to age-specific changes in expression of splicing factors (11). Studying temporal patterns of alternative splicing aberrations during neurodevelopment in IDs may be crucial to assess developmental delays and find critical periods to correct splicing deficits.

EFFECT OF ALTERNATIVE SPLICING ON BRAIN FUNCTION IN ID

Neuronal stimulation causes differential exon selection in mRNAs resulting in functionally diverse mRNA isoforms (47). Multiexon ion channel encoding mRNAs are among the most extensively spliced mRNAs in neuronal cells (48–54). Neuronal depolarization with KCl results in widespread alternative splicing changes in neuronal cultures *in-vitro*, affecting selective permeability of ion channels (33, 55, 56). Neuronal activity-dependent splicing changes may affect long-term synaptic plasticity. One example is activity-dependent splicing of RNAs encoding neurexins', a class of crucial trans-synaptic cell adhesion molecules that govern synapse assembly, transmission, and identity. Alternative splicing and alternative promoter usage result in thousands of neurexin (*Nrxn*) isoforms with potentially diverse functions (57). Membrane depolarization-induced shift in *Nrxn1* splice isoform choice via calcium/calmodulin-dependent kinase IV signaling altered trans-synaptic signaling regulated by SAM68 (a splicing factor) (56). Studies on alternative splicing in response to membrane depolarization in cerebellar neurons also identified SAM68 as a critical regulator of the splicing pattern (33). Neuronal activity-dependent alternative splicing is also highly dysregulated in ASD, specifically microexon (3–27 nt) splicing (15, 58). A mouse model mimicking microexon skipping in ASD for the translation initiation factor, eIF4G (Eukaryotic translation initiation factor 4 G), demonstrated activity-dependent exclusion of a microexon resulted in increased levels of synaptic proteins involved in mediating neuronal activity and synaptic plasticity (59).

Another neurodevelopmental disorder that shows prevalent alternative splicing changes that manifests with learning disabilities and autism is the Rett Syndrome (RTT) (12). RTT mainly occurs due to loss of the MeCP2 protein, which is studied using MeCP2 deficient mouse models. Widespread changes in alternative splicing were identified in MeCP2 null mice, which were exacerbated by membrane depolarization (14). Furthermore, MeCP2 null mice were more susceptible to seizures post-neuronal stimulation, which may be due to the altered splicing program. MeCP2 mediated alternative splicing may also regulate spatial learning-induced memory consolidation in hippocampal tissue (13). However, the contribution of alternative splicing defects in the MeCP2 null phenotype is still unclear (60). Highly congruent to ASD are the alternative splicing changes recently identified in the hippocampus from the Fragile X Syndrome mouse model [*Fmr1* knockout (KO)] (5). Synaptic vesicle localization and function were amongst the top enriched categories of differentially spliced genes. This could imply that, the synaptic plasticity deficits observed in *Fmr1* KO hippocampus tissue could be mediated by altered isoform ratios of key synaptic function genes.

MECHANISMS OF ALTERNATIVE DIFFERENTIAL SPLICING IN ID

Alternative splicing regulation is a highly variable process that depends on complex interactions between cis-acting splicing signals, splicing factor recruitment, RNA motifs, combinatorial action of RNA binding proteins, epigenetic marks on the chromatin, speed of polymerase movement, and other molecular features. The mechanisms contributing to alternative splicing in the mammalian nervous system, which have been reviewed

in Ref. (27), may explain the aberrant pre-mRNA splicing programs in IDs (**Figure 1**). Amongst the alternative splicing patterns examined in IDs, the most detailed have been in ASD. Studies from idiopathic ASD patient post-mortem brain tissue identified a reduction in nSR100/SRRM4 proteins in a significant proportion of tissue samples (6, 15). Mouse models mimicking haploinsufficiency of nSR100/SRRM4 (splicing regulators) also reproduced the misregulated splicing patterns seen in ASD patients and displayed deficits in social behavior (15). A study investigating alternative splicing defects in blood samples from autistic boys showed a set of 53 mis-spliced RNAs (18), several of which encode proteins that regulate differential alternative splicing. Altered expression of other splicing regulating factors, RBFOX1, RBFOX3, and PTBP1 have also been implicated in ASD (8, 16, 40, 41). Interestingly, mRNAs bound by RNA binding proteins, RBFOX1 and FMRP were differentially spliced in patient tissues from both ASD and Schizophrenia (8), suggesting that RNAs that show common splicing defects amongst these disorders may be targeted by similar processes.

Similar to ASD, a decrease in expression of splicing factor SRRM4 has also been identified in a PTEN mouse model along with decreased expression of other proteins such as NOVA2 and RBFOX1. Another hypothesis explaining the local splicing defects in PTEN, is that mutations in the *PTEN* gene might disrupt interactions of Pten with the spliceosomal protein U2AF2, thereby altering the local splicing program.

Alternative splicing of mRNAs can also be regulated by epigenetics, including histone modifications, DNA methylation, long non-coding RNAs posing as splicing factors, and potentially RNA modifications such as N6-methyladenosine (m6A), and DNA modifications such as 5-hydroxymethylcytosine (5hmC). In Rett syndrome, MeCP2-dependent spliced exons show differential epigenetic signatures based on their tendency of inclusion or exclusion. Excluded exons display the DNA modification 5hmC, and the presence of the histone modification H3K4me3 (histone H3 lysine 4 trimethylation) H3K4me3. Exons differentially included in the mature mRNA demonstrate enrichment of H3K36me3 (histone H3 lysine 36 trimethylation) marks in Mecp2-knockdown neurons (19). Similarly, epigenetic marks may contribute to the alternative splicing defects observed in brain tissues from the FXS mouse model. In FMRP deficient hippocampus tissues, one of the mRNAs released from translational repression is *SETD2*, resulting in increase of SETD2 (a lysine methyltransferase) protein levels. Increased SETD2 alters the H3k36me3 marks on the chromatin, which correlated with alternative splicing defects in mRNAs critical for proper synaptic function (5).

DISCUSSION

The functional significance of alternative splicing deficits in neurodevelopmental disorders is becoming increasingly clear owing to the ease of transcriptomic analysis and the use of mouse models mimicking exon mis-splicing events. An in-depth analysis of consolidated transcriptomic data from a large cohort of patients diagnosed with ASD, schizophrenia and bipolar disorder have provided significant insights enriching our understanding of the pathophysiology of psychiatric disorders (8). A key finding of this study was that rather than gene expression changes, isoform level aberrations were the most abundant, and demonstrated important functional gene category enrichments and disease specificity. Notably, the majority of the local splicing changes identified were restricted to each disorder, with only two genes that showed a splicing change in all three disorders. Amongst the pre-mRNA splicing aberrations identified in monogenic IDs, there is a significant overlap with those found in ASD patients, however majority of the splicing changes found are disorder specific. The process of differential alternative splicing resulting in dysregulated mRNA isoform expression maybe conserved amongst IDs, however, the exact genes may differ based on the different mechanisms that lead to the splicing deficits (**Table 1**; **Figure 1**). Thus, alternative splicing changes could potentially provide a molecular fingerprint to identify and differentiate among NDD pathologies. Growing numbers of studies have also identified differential pre-mRNA splicing in blood samples from patients (18, 20), suggesting the possibility of a potential biomarker for IDs and various NDDs. Given, the recent finding of differential splicing programs predominantly present in various IDs, a coordinated effort to generate and study large scale transcriptomics datasets will thus be beneficial. However, identifying all functional splicing changes in a given transcriptomics dataset is still a herculean task that relies primarily on identifying the protein domain encoded by the mis-spliced region. Considerations toward the extent of local splicing changes (ΔPSI, percent spliced-in) are imperative since the proportion of splicing change in isoforms, and the frequency of isoform expression, which often range from 10 to 40% in IDs, may not be enough to result in a functional outcome (protein expression or RNA localization). Whether a substantial ΔPSI change in one gene can result in a phenotype or smaller ΔPSI in multiple genes in the same pathway might contribute to disease manifestation would be helpful to understand. For example, although the ΔPSI of the differentially spliced mRNAs in the *Fmr1* KO mouse tissues were between 10 and 30%, most of the mRNAs were involved in synaptic vesicle recycling function in the hippocampus. Thus, the synaptic plasticity deficit in this tissue may in part be due to small splicing changes in many mRNAs in the same pathway. Furthermore, splicing analysis results may be subject to the choice of bioinformatic programs used to assess splicing changes, depth of sequencing, ΔPSI threshold, read length and type of sequencing, reference genome version, and sample characteristics such as age, tissue, and cell type studied. Consolidating alternative splicing patterns from different studies requires careful assessment of all factors that may contribute to a bias in data generation. Identification of similar alternative splicing changes in genes belonging to specific biological pathways such as synaptic function or brain development in different monogenic and complex neurological disorders, suggests that similar upstream regulatory pathways may be perturbed.

Dysregulated pre-mRNA splicing is a rapidly developing research focus in understanding pathophysiology of neurodevelopmental disorders. Several questions arise

based on current research and answering them is vital to our understanding of how neurodevelopmental disorders manifest and show such phenotypic diversity in patients. For example, is aberrant splicing a cause or consequence of changes in the epigenetic landscape, splicing factor levels, or RNA binding proteins? Can restoring alternative splicing defects result in reversing the course of the disorder? If not, can the alternative splicing changes be used as biomarkers to inform therapeutic strategies and disease progression? Can the alternative splicing perturbations explain commonalities and differences between IDs and therefore predict the severity of the disorder? The genome-wide perturbations of alternative pre-mRNA splicing is a recent finding, and it is of particular importance to specifically study isoform level gene regulation to fully understand the molecular mechanisms of neurodevelopmental disorders.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

The manuscript is written by SS. JR reviewed and provided substantial inputs to the manuscript.

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REFERENCES

1. Homberg JR, Kyzar EJ, Scattoni ML, Norton WH, Pittman J, Gaikwad S, et al. Genetic and environmental modulation of neurodevelopmental disorders: translational insights from labs to beds. *Brain Res Bull.* (2016) 125:79–91. doi: 10.1016/j.brainresbull.2016.04.015
2. Neul JL. The relationship of Rett syndrome and MECP2 disorders to autism. *Dialogues Clin Neurosci.* (2012) 14:253–62. doi: 10.31887/DCNS.2012.14.3/jneul
3. Swedo E, Idaikkadar N, Leemis R, Dias T, Radhakrishnan L, Stein Z, et al. Trends in U.S. emergency department visits related to suspected or confirmed child abuse and neglect among children and adolescents aged <18 years before and during the COVID-19 pandemic — United States, January 2019–September 2020. *MMWR Morb Mort Wkly Rep.* (2020) 69:1841–7. doi: 10.15585/mmwr.mm6949a1
4. Richter JD, Zhao X. The molecular biology of FMRP: new insights into Fragile X syndrome. *Nat Rev Neurosci.* (2021) 22:209–22. doi: 10.1038/s41583-021-00432-0
5. Shah S, Molinaro G, Liu B, Wang R, Huber KM, Richter JD. FMRP control of ribosome translocation promotes chromatin modifications and alternative splicing of neuronal genes linked to autism. *Cell Rep.* (2020) 30:4459–4472.e6. doi: 10.1101/801076
6. Irimia M, Weatheritt RJ, Ellis JD, Parikshak NN, Gonatopoulos-Pournatzis T, Babor M, et al. A highly conserved program of neuronal microexons is misregulated in autistic brains. *Cell.* (2014) 159:1511–23. doi: 10.1016/j.cell.2014.11.035
7. Quesnel-Vallières M, Irimia M, Cordes SP, Blencowe BJ. Essential roles for the splicing regulator nSR100/SRRM4 during nervous system development. *Genes Dev.* (2015) 29:746–59. doi: 10.1101/gad.256115.114
8. Gandal MJ, Zhang P, Hadjimichael E, Walker RL, Chen C, Liu S, et al. Transcriptome-wide isoform-level dysregulation in ASD, schizophrenia, and bipolar disorder. *Science.* (2018) 362:eaat8127. doi: 10.1126/science.aat8127
9. Reble E, Dineen A, Barr CL. The contribution of alternative splicing to genetic risk for psychiatric disorders. *Genes Brain Behav.* (2018) 17:1–12. doi: 10.1111/gbb.12430
10. Elorza A, Márquez Y, Cabrera JR, Sánchez-Trincado JL, Santos-Galindo M, Hernández IH, et al. Huntington's disease-specific mis-splicing unveils key effector genes and altered splicing factors. *Brain.* (2021) 144:2009–23. doi: 10.1093/brain/awab087
11. Thacker S, Sefyi M, Eng C. Alternative splicing landscape of the neural transcriptome in a cytoplasmic-predominant Pten expression murine model of autism-like Behavior. *Transl Psychiatry.* (2020) 10:380. doi: 10.1038/s41398-020-01068-x
12. Li R, Dong Q, Yuan X, Zeng X, Gao Y, Chiao C, et al. Misregulation of alternative splicing in a mouse model of Rett syndrome. *PLoS Genet.* (2016) 12:e1006129. doi: 10.1371/journal.pgen.1006129
13. Brito DVC, Gulmez Karaca K, Kupke J, Frank L, Oliveira AMM. MeCP2 gates spatial learning-induced alternative splicing events in the mouse hippocampus. *Mol Brain.* (2020) 13:1–16. doi: 10.1186/s13041-020-00695-1
14. Osenberg S, Karten A, Sun J, Li J, Charkowick S, Felice CA, et al. Activity-dependent aberrations in gene expression and alternative splicing in a mouse model of Rett syndrome. *Proc Natl Acad Sci USA.* (2018) 115:E5363–72. doi: 10.1073/pnas.1722546115
15. Quesnel-Vallières M, Dargaei Z, Irimia M, Gonatopoulos-Pournatzis T, Ip JY, Wu M, et al. Misregulation of an activity-dependent splicing network as a common mechanism underlying autism spectrum disorders. *Mol Cell.* (2016) 64:1023–34. doi: 10.1016/j.molcel.2016.11.033
16. Li YI, Sanchez-Pulido L, Haerty W, Ponting CP. RBFOX and PTBP1 proteins regulate the alternative splicing of micro-exons in human brain transcripts. *Genome Res.* (2015) 25:1–13. doi: 10.1101/gr.181990.114
17. Parikshak NN, Swarup V, Belgard TG, Irimia M, Ramaswami G, Gandal MJ, et al. Genome-wide changes in lncRNA, splicing, and regional gene expression patterns in autism. *Nature.* (2016) 560:E30. doi: 10.1038/nature20612
18. Stamova BS, Tian Y, Nordahl CW, Shen MD, Rogers S, Amaral DG, et al. Evidence for differential alternative splicing in blood of young boys with autism spectrum disorders. *Mol Autism.* (2013) 4:1–16. doi: 10.1186/2040-2392-4-30
19. Cheng TL, Chen J, Wan H, Tang B, Tian W, Liao L, et al. Regulation of mRNA splicing by MeCP2 via epigenetic modifications in the brain. *Sci Rep.* (2017) 7:1–12. doi: 10.1038/srep42790
20. Glatt S, Chandler S, Bousman C, Chana G, Lucero G, Tatro E, et al. Alternatively spliced genes as biomarkers for schizophrenia, bipolar disorder and psychosis: a blood-based spliceome-profiling exploratory study (Supplementry Table). *Curr Pharmacogenomics Person Med.* (2012) 7:164–88. doi: 10.2174/1875692110907030164
21. Lin L, Park JW, Ramachandran S, Zhang Y, Tseng YT, Shen S, et al. Transcriptome sequencing reveals aberrant alternative splicing in Huntington's disease. *Hum Mol Genet.* (2016) 25:3454–66. doi: 10.1093/hmg/ddw187
22. Labadorf AT, Myers RH. Evidence of extensive alternative splicing in post mortem human brain HTT transcription by mRNA sequencing. *PLoS ONE.* (2015) 10:e0141298. doi: 10.1371/journal.pone.0141298
23. Schilling J, Broemer M, Atanassov I, Duernberger Y, Vorberg I, Dieterich C, et al. deregulated splicing is a major mechanism of RNA-induced toxicity in huntington's disease. *J Mol Biol.* (2019) 431:1869–77. doi: 10.1016/j.jmb.2019.01.034
24. Chen T, Zhang B, Ziegenhals T, Prusty AB, Fröhler S, Grimm C, et al. A missense mutation in SNRPE linked to non-syndromal microcephaly interferes with U snRNP assembly and pre-mRNA splicing. *PLoS Genet.* (2019) 15:e1008460. doi: 10.1371/journal.pgen.1008460
25. Gueroussov S, Gonatopoulos-Pournatzis T, Irimia M, Raj B, Lin J-Y, Gingras A-C, et al. An alternative splicing event amplifies evolutionary differences between vertebrates. *Science.* (2015) 349:868–73. doi: 10.1126/science.aaa8381

26. Wang ET, Sandberg R, Luo S, Khrebtukova I, Zhang L, Mayr C, et al. Alternative isoform regulation in human tissue transcriptomes. *Nature.* (2008) 456:470–6. doi: 10.1038/nature07509
27. Raj B, Blencowe BJ. alternative splicing in the mammalian nervous system: recent insights into mechanisms and functional roles. *Neuron.* (2015) 87:14–27. doi: 10.1016/j.neuron.2015.05.004
28. Parras A, Anta H, Santos-Galindo M, Swarup V, Elorza A, Nieto-González JL, et al. Autism-like phenotype and risk gene mRNA deadenylation by CPEB4 mis-splicing. *Nature.* (2018) 560:441–6. doi: 10.1038/s41586-018-0423-5
29. Bill BR, Lowe JK, DyBuncio CT, Fogel BL. Orchestration of neurodevelopmental programs by RBFOX1: implications for autism spectrum disorder. *Int Rev Neurobiol.* (2013) 113:251–67. doi: 10.1016/B978-0-12-418700-9.00008-3
30. Jaganathan K, Kyriazopoulou Panagiotopoulou S, McRae JF, Darbandi SF, Knowles D, Li YI, et al. predicting splicing from primary sequence with deep learning. *Cell.* (2019) 176:535–48.e24. doi: 10.1016/j.cell.2018.12.015
31. Kasherman MA, Premarathne S, Burne THJ, Wood SA, Piper M. The ubiquitin system: a regulatory hub for intellectual disability and autism spectrum disorder. *Mol Neurobiol.* (2020) 57:2179–93. doi: 10.1007/s12035-020-01881-x
32. Fertuzinhos S, Li M, Kawasawa YI, Ivic V, Franjic D, Singh D, et al. Laminar and temporal expression dynamics of coding and noncoding RNAs in the mouse neocortex. *Cell Rep.* (2014) 13:938–50. doi: 10.1016/j.celrep.2014.01.036
33. Farini D, Cesari E, Weatheritt RJ, La Sala G, Naro C, Pagliarini V, et al. A dynamic splicing program ensures proper synaptic connections in the developing cerebellum. *Cell Rep.* (2020) 31:107703. doi: 10.1016/j.celrep.2020.107703
34. Thompson M, Bixby R, Dalton R, Vandenburg A, Calarco JA, Norris AD. Splicing in a single neuron is coordinately controlled by RNA binding proteins and transcription factors. *Elife.* (2019) 8:1–19. doi: 10.7554/eLife.46726
35. Iijima T, Iijima Y, Witte H, Scheiffele P. Neuronal cell type-specific alternative splicing is regulated by the KH domain protein SLM1. *J Cell Biol.* (2014) 204:331–42. doi: 10.1083/jcb.201310136
36. Li Q, Zheng S, Han A, Lin C-H, Stoilov P, Fu X-D, et al. The splicing regulator PTBP2 controls a program of embryonic splicing required for neuronal maturation. *Elife.* (2014) 3:1–24. doi: 10.7554/eLife.01201
37. Zhang M, Ergin V, Lin L, Stork C, Chen L, Zheng S. Axonogenesis is coordinated by neuron-specific alternative splicing programming and splicing regulator PTBP2. *Neuron.* (2019) 101:690–706.e10. doi: 10.1016/j.neuron.2019.01.022
38. Weyn-Vanhentenryck SM, Feng H, Ustianenko D, Duffié R, Yan Q, Jacko M, et al. Precise temporal regulation of alternative splicing during neural development. *Nat Commun.* (2018) 9:2189. doi: 10.1038/s41467-018-04559-0
39. Fugier C, Klein AF, Hammer C, Vassilopoulos S, Ivarsson Y, Toussaint A, et al. Misregulated alternative splicing of BIN1 is associated with T tubule alterations and muscle weakness in myotonic dystrophy. *Nat Med.* (2011) 17:720–5. doi: 10.1038/nm.2374
40. Lee JA, Damianov A, Lin CH, Fontes M, Parikshak NN, Anderson ES, et al. Cytoplasmic Rbfox1 regulates the expression of synaptic and autism-related genes. *Neuron.* (2016) 89:113–28. doi: 10.1016/j.neuron.2015.11.025
41. Weyn-Vanhentenryck SM, Mele A, Yan Q, Sun S, Farny N, Zhang Z, et al. HITS-CLIP and integrative modeling define the Rbfox splicing-regulatory network linked to brain development and autism. *Cell Rep.* (2014) 6:1139–52. doi: 10.1016/j.celrep.2014.02.005
42. Gonatopoulos-Pournatzis T, Blencowe BJ. Microexons: at the nexus of nervous system development, behaviour and autism spectrum disorder. *Curr Opin Genet Dev.* (2020) 65:22–33. doi: 10.1016/j.gde.2020.03.007
43. Raj T, Li YI, Wong G, Humphrey J, Wang M, Ramdhani S, et al. Integrative transcriptome analyses of the aging brain implicate altered splicing in Alzheimer's disease susceptibility. *Nat Genet.* (2018) 50:1584–92. doi: 10.1038/s41588-018-0238-1
44. Stilling RM, Benito E, Gertig M, Barth J, Capece V, Burkhardt S, et al. De-regulation of gene expression and alternative splicing affects distinct cellular pathways in the aging hippocampus. *Front Cell Neurosci.* (2014) 8:1–15. doi: 10.3389/fncel.2014.00373
45. Deschênes M, Chabot B. The emerging role of alternative splicing in senescence and aging. *Aging Cell.* (2017) 16:918–33. doi: 10.1111/acel.12646
46. Tollervey JR, Wang Z, Hortobágyi T, Witten JT, Zarnack K, Kayikci M, et al. Analysis of alternative splicing associated with aging and neurodegeneration in the human brain. *Genome Res.* (2011) 21:1572–82. doi: 10.1101/gr.122226.111
47. Thalhammer A, Jaudon F, Cingolani LA. Emerging roles of activity-dependent alternative splicing in homeostatic plasticity. *Front Cell Neurosci.* (2020) 14:104. doi: 10.3389/fncel.2020.00104
48. Copley RR. Evolutionary convergence of alternative splicing in ion channels. *Trends Genet.* (2004) 20:171–6. doi: 10.1016/j.tig.2004.02.001
49. Babini E, Paukert M, Geisler HS, Gründer S. Alternative splicing and interaction with di- and polyvalent cations control the dynamic range of acid-sensing ion channel 1 (ASIC1). *J Biol Chem.* (2002) 277:41597–603. doi: 10.1074/jbc.M205877200
50. Staudacher K, Baldea I, Kisselbach J, Staudacher I, Rahm AK, Schweizer PA, et al. Alternative splicing determines mRNA translation initiation and function of human K 2P10.1 K + channels. *J Physiol.* (2011) 589:3709–20. doi: 10.1113/jphysiol.2011.210666
51. Xie J, Black DL. A CaMK IV responsive RNA element mediates depolarization-induced alternative splicing of ion channels. *Nature.* (2001) 410:936–9. doi: 10.1038/35073593
52. Allen SE, Toro CP, Andrade A, López-Soto EJ, Denome S, Lipscombe D. Cell-specific RNA binding protein Rbfox2 regulates CaV2.2 mRNA exon composition and CaV2.2 current size. *eNeuro.* (2017) 4:ENEURO.0332-16.2017. doi: 10.1523/ENEURO.0332-16.2017
53. Heinzen EL, Yoon W, Weale ME, Sen A, Wood NW, Burke JR, et al. Alternative ion channel splicing in mesial temporal lobe epilepsy and Alzheimer's disease. *Genome Biol.* (2007) 8:R32. doi: 10.1186/gb-2007-8-3-r32
54. Javier E, Soto L, Lipscombe D. Cell-specific exon methylation and CTCF binding in neurons regulates calcium ion channel splicing and function. *eLife.* (2020) 9:e54879. doi: 10.7554/eLife.54879
55. An P, Grabowski PJ. Exon silencing by UAGG motifs in response to neuronal excitation. *PLoS Biol.* (2007) 5:e36. doi: 10.1371/journal.pbio.0050036
56. Iijima T, Wu K, Witte H, Hanno-Iijima Y, Glatter T, Richard S, et al. SAM68 regulates neuronal activity-dependent alternative splicing of neurexin-1. *Cell.* (2011) 147:1601–14. doi: 10.1016/j.cell.2011.11.028
57. Smith RM, Sadee W. Synaptic signaling and aberrant RNA splicing in autism spectrum disorders. *Front Synaptic Neurosci.* (2011) 3:1. doi: 10.3389/fnsyn.2011.00001
58. Quesnel-Vallières M, Weatheritt RJ, Cordes SP, Blencowe BJ. Autism spectrum disorder: insights into convergent mechanisms from transcriptomics. *Nat Rev Genet.* (2019) 20:51–63. doi: 10.1038/s41576-018-0066-2
59. Gonatopoulos-Pournatzis T, Niibori R, Salter EW, Weatheritt RJ, Tsang B, Farhangmehr S, et al. Autism-misregulated eIF4G microexons control synaptic translation and higher order cognitive functions. *Mol Cell.* (2020) 77:1176–92.e16. doi: 10.1016/j.molcel.2020.01.006
60. Chhatbar K, Cholewa-Waclaw J, Shah R, Bird A, Sanguinetti G. Quantitative analysis questions the role of MeCP2 as a global regulator of alternative splicing. *PLoS Genet.* (2020) 16:1–14. doi: 10.1371/journal.pgen.1009087

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frontiers in Neuroscience

CASE REPORT
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Case Report: Coexistence of Alzheimer-Type Neuropathology in Fragile X-Associated Tremor Ataxia Syndrome

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This case documents the co-occurrence of the fragile X-associated tremor ataxia syndrome (FXTAS) and Alzheimer-type neuropathology in a 71-year-old premutation carrier with 85 CGG repeats in the fragile X mental retardation 1 (*FMR1)* gene, in addition to an apolipoprotein E (*APOE)* ε4 allele. FXTAS and Alzheimer's Disease (AD) are late-onset neurodegenerative diseases that share overlapping cognitive deficits including processing speed, working memory and executive function. The prevalence of coexistent FXTAS-AD pathology remains unknown. The clinical picture in this case was marked with rapid cognitive decline between age 67 and 71 years in addition to remarkable MRI changes. Over the 16 months between the two clinical evaluations, the brain atrophied 4.12% while the lateral ventricles increased 26.4% and white matter hyperintensities (WMH) volume increased 15.6%. Other regions atrophied substantially faster than the whole brain included the thalamus (−6.28%), globus pallidus (−10.95%), hippocampus (−6.95%), and amygdala (−7.58%). A detailed postmortem assessment included an MRI with confluent WMH and evidence of cerebral microbleeds (CMB). The histopathological study demonstrated FXTAS inclusions in neurons and astrocytes, a widespread presence of phosphorylated tau protein and, amyloid β plaques in cortical areas and the hippocampus. CMBs were noticed in the precentral gyrus, middle temporal gyrus, visual cortex, and brainstem. There were high amounts of iron deposits in the globus pallidus and the putamen consistent with MRI findings. We hypothesize that coexistent FXTAS-AD neuropathology contributed to the steep decline in cognitive abilities.

Keywords: FXTAS, Alzheimer-type dementia, *FMR1* gene, CGG expansion, *APOE* ε4 allele, neurodegeneration, cognitive decline

INTRODUCTION

Fragile X-associated tremor/ataxia syndrome (FXTAS) is a late-onset neurodegenerative disease characterized by neurological involvement including cerebellar ataxia, intention tremor, neuropathy, parkinsonism, executive dysfunction, and cognitive deficits. Clinical symptoms usually become apparent between the sixth and seventh decade of life. Carrying the premutation (55–200 CGG repeats) in the *FMR1* gene located in the X chromosome confers the genetic background for the development of FXTAS in ∼40% of male and 16% of female carriers (Hagerman and Hagerman, 2016). Most of the affected patients face a slow but steady progression of motor and cognitive deficits. During the disease's final stages, ∼15–25 years after the onset of symptoms, the patients are unable to perform basic activities of daily living and lose their independence. Cognitive decline leading to dementia is seen in ∼50% of male patients with FXTAS (Seritan et al., 2016). Prior reports have introduced epigenetic/environmental risk factors related to an early presentation, greater severity of symptoms or faster progression of FXTAS including chronic use of addictive substances (Martinez-Cerdeno et al., 2015, Muzar et al., 2014, Muzar et al., 2015), anesthetics/general surgery (Ligsay et al., 2019), and exposure to environmental neurotoxins (Saldarriaga et al., 2019). Here we report an unusual case with a dramatic progression of cognitive decline associated with disinhibition in a patient with FXTAS and an *APOE* ε4 allele. The patient died within 10 years of clinical involvement. He was evaluated clinically, and he had postmortem imaging and tissue examination demonstrating co-occurrence of Alzheimer changes.

CASE DESCRIPTION

The patient initially presented to us at age 67 with a history of both tremor and balance difficulties. He was confirmed to have 85 CGG repeats and 4 daughters with the *FMR1* premutation as well as a niece and nephew with fragile X syndrome. Before developing FXTAS, he was noted to be athletic and an extremely successful professional.

After the loss of his father, at age 61, he developed bereavement-related depression, however, he did not receive antidepressants nor did he have therapy or counseling for his depression. Soon after, he began to present short-term memory problems, motor deficits including tremor and balance problems, as well as neuropathy in his lower extremities. As his hand tremor worsened, he also developed a head tremor. By age 62, his worsening short-term memory forced him to retire from his business. He began to have long lapses in his conversations as his word retrieval became worse. He had an episode of wandering at age 67 away from his hotel and he was disoriented, a subsequent evaluation with a CAT scan was normal. He also had frequent falls and at age 69 he fell but he did not have significant head trauma, he was discharged after neurological evaluation and observation in the emergency room.

His past medical history included episodes of vertigo which began at age 57. These episodes usually lasted a few minutes except for two episodes that were associated with muscle weakness and lasted a day. He also had a history of atrial fibrillation (Afib) diagnosed at age 57, requiring cardioversion at presentation. Recurrent Afib was treated with propafenone 150 mg bid and warfarin. Additional medications included pravastatin 20 mg a day, vitamins, and antioxidants. He also had 2 hospitalizations for severe pneumonia.

On his initial exam at age 67, his hearing was mildly decreased bilaterally, and his smile was asymmetrical with less tone on the left. His finger joints were swollen, and he had arthritic changes and nodules on the upper aspect of his fingers. His reflexes were hypoactive and absent at the ankles and vibration sense was diminished in the feet. He had prominent primitive reflexes including grasp, glabellar, snout and palmomental reflexes. He had an intention tremor bilaterally, a hand positional tremor bilaterally, and a mild "yes yes" head tremor. He had significant dysdiadochokinesis bilaterally and was unable to tandem walk with significant ataxia on heel to shin movements. Cognitive testing is shown in **Table 1**. Treatment with memantine and donepezil, and a follow-up with a geriatric psychiatrist and physical therapy was recommended for ataxia.

He was followed 2 years later, at age 69, with worsened tremors and swallowing problems. Although he was treated with memantine and donepezil his cognition had worsened. He had become more disinhibited in public and had aggressive behavior at night, so he was started on quetiapine to aid in sleep.

On exam, he had a coarse intention, positional and resting tremor in addition to his head tremor. He had masked facies, bradykinesia, and a cervical dystonia with his head tilted to the left. His reflexes were decreased and absent in the ankles and he continued to have signs of neuropathy with decreased pinprick and vibration sensations. He had marked primitive reflexes as previously noted. Cognitive testing (**Table 1**) demonstrated a major neurocognitive disorder and the results from the Structured Clinical Interview for DSM-IV (SCID) diagnosed Social Phobia (F40.1, 300.23). A trial with carbidopa/levodopa for emerging parkinsonian features was recommended. A comprehensive review of family history at this time revealed a maternal uncle who died from AD in his 50s; based on this new information genetic testing for *APOE* was carried out to evaluate the patient's predisposition for AD.

Although carbidopa/levodopa was initially beneficial, over the next 2 years he gradually developed worsening of his symptoms of tremor, weakness and falling until he was bedridden. He had episodes of staring which were thought to be transient ischemic attacks, but an electroencephalogram demonstrated right and left temporal spikes and a disorganized background rhythm. Due to swallowing problems, he was hospitalized with aspiration pneumonia at age 71. A percutaneous endoscopic gastrostomy (PEG) tube was placed for nutrition. An echocardiogram taken at the time of hospitalization showed mild left ventricular hypertrophy and tricuspid regurgitation as well as a small pericardial effusion. He was eventually transferred to hospice and needed continuous oxygen supply before he passed away at age 71.

TABLE 1 | Summary of neuropsychiatric results.

		2016 Score	2016 Percentile rank	2017 Score	2017 Percentile rank	Description
WAIS-IV						
	Verbal IQ	70	2	54	0.1	Borderline (2016), Extremely Low (2017)
	Performance IQ	65	1	63	1	Extremely Low
	Full Scale IQ	62	1	51	0.1	Extremely Low
	VCI	70	2	54	0.1	Extremely Low
	PRI	65	1	63	1	Extremely Low
	WMI	71	3	53	0.1	Extremely Low
	PSI	65	1	59	0.3	Extremely Low
WMS-IV*						
	Auditory Memory	–	–	58	0.3	Extremely Low
	Visual Memory	–	–	45	<0.1	Extremely Low
	Immediate Memory	–	–	54	0.1	Extremely Low
	Delayed Memory	–	–	54	0.1	Extremely Low
BDS-2						
		–	–	2	–	Severe difficulties with executive functioning
MMSE						
		–	–	15	–	Significant impairment in cognitive functioning

**WMS Scores are provided as Scaled scores.*

Based on the neuropsychological results and the caregiver's report on the patient's decline, a diagnosis of Major Neurocognitive Disorder Due to Another Medical Condition (F02.80, 294.10, DSM- 5) was probable. WAIS-IV, Wechsler Adult Intelligence Scale, 4th Edition; VCI, Verbal Comprehension Index; PCI, Perceptual Reasoning Index; WMI, Working Memory Index; PSI, Processing Speed Index; WMS-IV, Wechsler Memory Scale, 4th Edition; BDS-2, Behavior Dyscontrol Scale 2nd Edition; MMSE, Mini-Mental-State Exam.

DIAGNOSTIC ASSESSMENT

Neuropsychiatric Assessment

Cognitive testing is listed in **Table 1** (WAIS-IV, Wechsler Adult Intelligence Scale, 4th Edition)(Wechsler, 2008). An assessment of memory skills (WMS-IV, Wechsler Memory Scale, 4th Edition)(Wechsler, 2009) and several other neuropsychological assessments were also conducted (**Table 1**) to evaluate executive function and general cognitive status [Behavior Dyscontrol Scale 2nd Edition(BDS-2) (Grigsby and Kaye, 1996), Mini-Mental-State Exam (MMSE)] (Folstein et al., 1975). His memory and executive function abilities demonstrated significant decline over the 2 clinical visits. The examiner noted that he often stared blankly during the second assessment, and it was not fully clear whether he understood the questions properly.

Neuroimaging

Both *in vivo* and postmortem MRI were acquired on a Siemens Trio 3T MRI scanner (Siemens Medical Solutions, Erlangen, Germany) using a 32-channel headcoil. For postmortem MRI, the right hemisphere was immersed in an inert proton-free fluid, 3M fluorinert electronic liquid (FC-770, Parallax Technology, Inc.) during the acquisitions of T2 and multi-echo T2* images.

Brain MRI during the initial evaluation showed a large middle cerebellar peduncles (MCP) sign, thin corpus callosum and white matter disease (WMD) in the splenium and genu of his corpus callosum and WMD in the right temporal lobe and periventricular areas (**Figures 1A,D,G,I**). Brain imaging at follow-up demonstrated severe brain atrophy with remarkable asymmetrical atrophy of the temporal lobes. Worsening of WMD in all areas including the insula and periventricular regions. The ventricles were larger and the corpus callosum was thinner when compared to the MRI taken 16 months prior (**Figures 1B,E**).

A quantitative analysis of brain atrophy was performed using volBrain software pipeline that implements a multi-atlas label fusion technique for segmenting the intracranial cavity, brain, lateral ventricle, cerebellar lobules, and subcortical nuclei (Manjon et al., 2014; Manjon and Coupe, 2016; Romero et al., 2017) (**Figures 1D,E,I,J**). Age- and sex-specific normative data are also available (Coupe et al., 2017). White matter hyperintensities (WMHs) were segmented automatically on fluid attenuated inversion recovery (FLAIR) scans using the LST toolbox from SPM12 (Schmidt et al., 2012), followed by manual editing using ITKsnap (Weis et al., 1993).

Compared with age- and sex-specific normative data (Coupe et al., 2017), percentages of intracranial volume (ICV) for whole brain, whole brain white matter, cerebrum, cerebral white matter, brainstem, thalamus, and amygdala were lower than the 95% lower limits while those of cerebrospinal fluid (CSF) and lateral ventricles were above the 95% upper limits for both visits. The ICV percentages of other regions, including the gray matter, cerebrum gray matter, cerebellum, cerebellar gray matter, and other nuclei under the investigation (**Table 2**) were within the 95% limits for both visits.

Over the 16 months between the two visits, the brain atrophied 4.12% while the lateral ventricles increased 26.4% and WMH volume increased 15.6%. Other regions atrophied substantially faster than the whole brain included the thalamus (−6.28%), globus pallidus (−10.95%), hippocampus

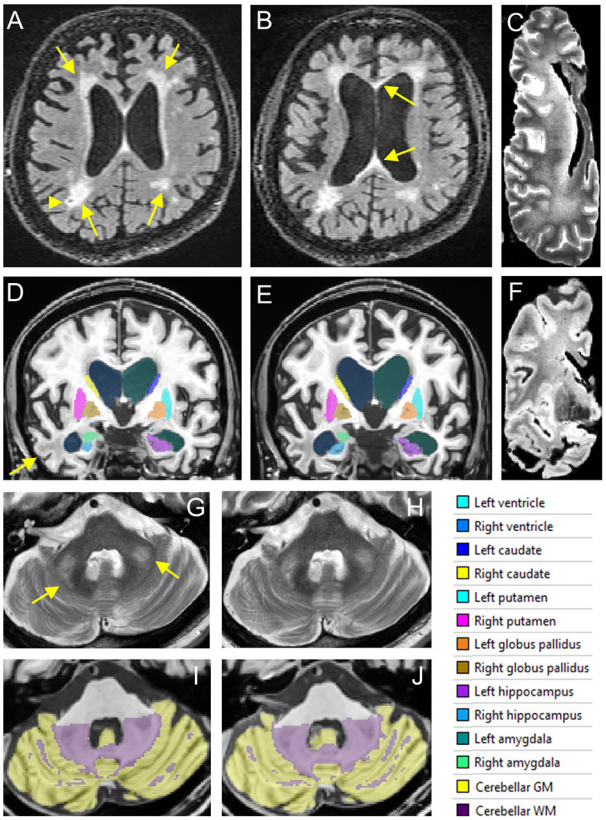

FIGURE 1 | *In vivo* and postmortem MRI showing white matter lesions, brain atrophy, and enlarged ventricles. **(A–C)** FLAIR scans acquired in 2016 **(A)** and 2017 **(B)** show periventricular WMHs (arrows in **A**) and a lacune (arrowhead) in right parietal white matter. The corpus callosum is thinned and has WMHs in both genu and splenium (arrows in **B**). WMHs become confluent in the frontal and parietal regions in the postmortem T2 scan of the right hemisphere **(C)**. **(D–F)** Segmentation of the lateral ventricles and subcortical nuclei on T1 scans acquired in 2016 **(D)** and 2017 **(E)**. White matter lesions in the right temporal lobe are observed on both *in vivo* MRI scans (arrow) as well as the postmortem T2* scan **(F)**. **(G,H)** The MCP sign (arrows) on T2 scans acquired in 2016 **(G)** and 2017 **(H)**. **(I,J)** Segmentation of the cerebellum on T1scans acquired in 2016 **(I)** and 2017 **(J)**.

(−6.95%), and amygdala (−7.58%). In contrast, the cerebellum (−2.40%), brainstem (−2.37%), caudate (−1.76%), and putamen (−1.03%) experienced slower than average atrophy rate (**Table 2**).

Post-mortem MRI showed confluent WMHs in the frontal and parietal lobes on T2 scan (**Figure 1C**) indicating further WMH expansion during the last 2 years of life and tissue loss in anterior temporal white matter (**Figure 1F**). Transverse relaxation rate, R2*, sensitive to iron content in the tissue, was estimated by fitting an ordinary lease squares function to the signal intensities of the multi-echo T2* scan (Peran et al., 2007). Small focal increases in R2* signals, consistent with cerebral microbleeds (CMBs), were observed in cerebral white matter as well as increased R2* signals in the deep nuclei such as the putamen, globus pallidus, red nucleus, substantia nigra (SN), and cerebellar dentate nucleus.

Genetic Evaluation

DNA testing, including PCR and Southern blot analysis, as previously described (Filipovic-Sadic et al., 2010, Tassone et al., 2008), revealed the presence of a premutation allele of 85 CGG repeats. *FMR1* mRNA expression levels, measured by real time qRT-PCR (Tassone et al., 2000), were 2.5-fold higher than normal (2.58 StErr 0.04) (**Figure 2**). Genotyping for *APOE* allelic variants were carried out using TaqMan® assay genotype. An *APOE* ε3/ε4 variant was documented.

Postmortem Brain Assessment

The right hemisphere was fixed in 10% formalin for 8 weeks before dissection. After fixation gross descriptions were taken and the right hemisphere was coronally sectioned. Gross anatomy showed atrophy in the frontal and temporal lobes (**Figure 3A**), as well as mild ventricular enlargement, more predominant posteriorly. No evidence of major haemorrhagic strokes, infarctions, or abnormal growth were found grossly. The SN was well pigmented. Evidence of mild atherosclerosis was found within the basilar artery (**Figure 3A**).

Upon coronal sectioning, select anatomic areas were sampled from the frontal cortex, basal ganglia, inferior parietal cortex, temporal cortex, hippocampal formation including CA1, CA2 and entorhinal cortex, occipital cortex (Brodmann areas 17/18), hemisection of midbrain including SN and cerebellum, and then immersed in 30% sucrose in Tris-buffered saline (TBS). Once saturated, samples were embedded in optimal cutting temperature (OCT) compound (Fisher,USA), and frozen at −80°C. Sections were cut in cryostat at 14 μm thickness.

Our sampling deviated from the current recommendation for the evaluation of AD changes (Montine et al., 2012); since we did not include the middle frontal gyrus and the superior temporal gyrus in our analysis. In addition, we did not use the Consortium to Establish a Registry for AD (CERAD) (Mirra et al., 1991) protocol for neuritic plaque scoring. Hematoxylin and eosin stain was used for the assessment of WMD and CMBs, recently described as a common pathologic feature of FXTAS (Salcedo-Arellano et al., 2021).

Prior to immunostaining, slides were washed in TBS, hydrated in ethanol 100–50%, followed by antigen retrieval and incubation in 3% hydrogen peroxide, permeabilized and blocked in a TBS based solution (75% TBS, 15% Triton, 10% serum) for 2 h. Rabbit anti-ubiquitin (1:150; Dako, Glostrup, Denmark) was used to identify intranuclear inclusions commonly described in neurons and astrocytes, the major pathologic hallmark of FXTAS. A mouse monoclonal anti-phosphorylation clone AT8 tau [1:200; Invitrogen MN 1020 (Ser202, Thr205)] and a purified mouse anti-α-Synuclein, pY125 (1:200; BD Pharmingen) were used to evaluate tau aggregates and to confirm the presence of Lewy bodies in the SN respectively. A polyclonal rabbit anti-β amyloid 1–42 (ab10148, 1:100; Abcam, Cambridge, UK) was used to evaluate amyloid plaques and cerebral amyloid angiopathy (CAA), slides were incubated overnight at −4°C. Pearls Prussian blue (Meguro et al., 2007), was completed for the assessment of iron bound to hemosiderin, known to correspond to chronic microhaemorrhages and iron deposits.

TABLE 2 | Percentages of change and brain regional volumes compared with age- and sex-specific normative data.

Brain regions	Visit 1 (Age 67.9)						Visit 2 (Age 69.2)						Whole brain % of change
	Volume (cm^3)	% of ICV	95% lower limit	95% upper limit	Left hemi. volume (cm^3)	Right hemi. volume (cm^3)	Volume (cm^3)	% of ICV	95% lower limit	95% upper limit	Left hemi. volume (cm^3)	Right hemi. volume (cm^3)	
Whole brain	947	**63.4**	75.8	86.4	–	–	908	**61.3**	75.1	85.7	–	–	−4.1
Gray matter	663	44.4	40.0	51.3	–	–	635	42.9	39.9	51.2	–	–	−4.2
White matter	283	**19.0**	29.3	41.6	–	–	272	**18.4**	28.7	41.0	–	–	−3.9
Cerebrum	814	**54.5**	65.3	75.4	**416**	**397**	779	**52.5**	64.7	74.8	**399**	**379**	−4.3
Gray matter	544	36.4	33.6	43.2	276	268	523	35.3	33.5	43.0	266	257	−4.0
White matter	269	**18.0**	26.6	37.4	**140**	**129**	256	**17.3**	26.1	36.9	**134**	**122**	−5.0
Cerebellum	114	**7.6**	7.8	10.3	**56.2**	57.8	111	**7.5**	7.7	10.3	**54.0**	**56.5**	−3.1
Gray matter	102	6.8	5.6	8.2	50.4	51.8	98	6.6	5.6	8.2	48.2	49.9	−4.0
White matter	11.9	**0.79**	1.3	3.0	**5.8**	**6.1**	12.4	**0.84**	1.2	3.0	**5.9**	**6.6**	4.8
Brainstem	19.1	**1.3**	1.4	1.9	–	–	18.7	**1.3**	1.4	1.9	–	–	−2.4
CSF	546	**36.6**	13.6	24.2	–	–	574	**38.7**	14.3	24.9	–	–	5.1
Lateral ventricles	78	**5.2**	0.60	3.04	**37.8**	**40.1**	98	**6.6**	0.72	3.16	**47.4**	**51.1**	26.4
Caudate	5.49	0.37	0.36	0.55	2.81	**2.67**	5.39	0.36	0.36	0.55	2.80	**2.60**	−1.8
Putamen	7.16	0.48	0.42	0.64	3.50	3.66	7.09	0.48	0.42	0.63	3.50	3.59	−1.0
Thalamus	7.05	**0.47**	0.59	0.79	**3.47**	**3.57**	6.60	**0.45**	0.58	0.78	**3.22**	**3.38**	−6.3
Globus pallidus	2.30	0.15	0.13	0.20	1.19	1.11	2.05	0.14	0.13	0.20	1.08	0.97	−11.0
Hippocampus	6.96	0.47	0.44	0.63	3.50	3.46	6.48	0.44	0.44	0.62	3.25	3.23	−6.9
Amygdala	0.99	**0.07**	0.09	0.14	**0.53**	**0.46**	0.91	**0.06**	0.09	0.14	**0.48**	**0.44**	−7.6
WMHs	44	2.94	–	–	–	–	51	3.42	–	–	–	–	15.5

Bold, outside of the 95% limit of the age- and sex-specific normative data.

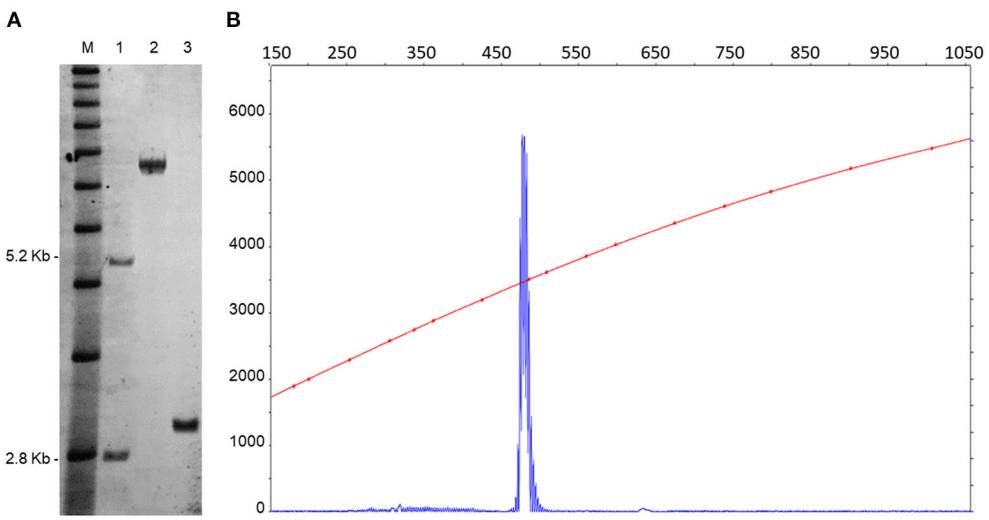

FIGURE 2 | CGG repeat size and methylation status were determined by a combination of Southern blots **(A)** and capillary electrophoresis **(B)** on genomic DNA isolated from a female, negative control (lane 1), from a full mutation control (>200 CGG repeats) (lane 2). The SB analysis demonstrates the presence of an unmethylated premutation alleles in the proband (lane 3). M = DNA marker, 1 kb ladder. Normal unmethylated band (2.8 kb) and normal methylated band (5.2 kb) shown on the left. The electrophoregram **(B)** shows the presence of a single peak representing the premutation allele. The X-axis indicates the size of the allele in base pairs and the Y-axis indicates the fluorescence intensity of each allele.

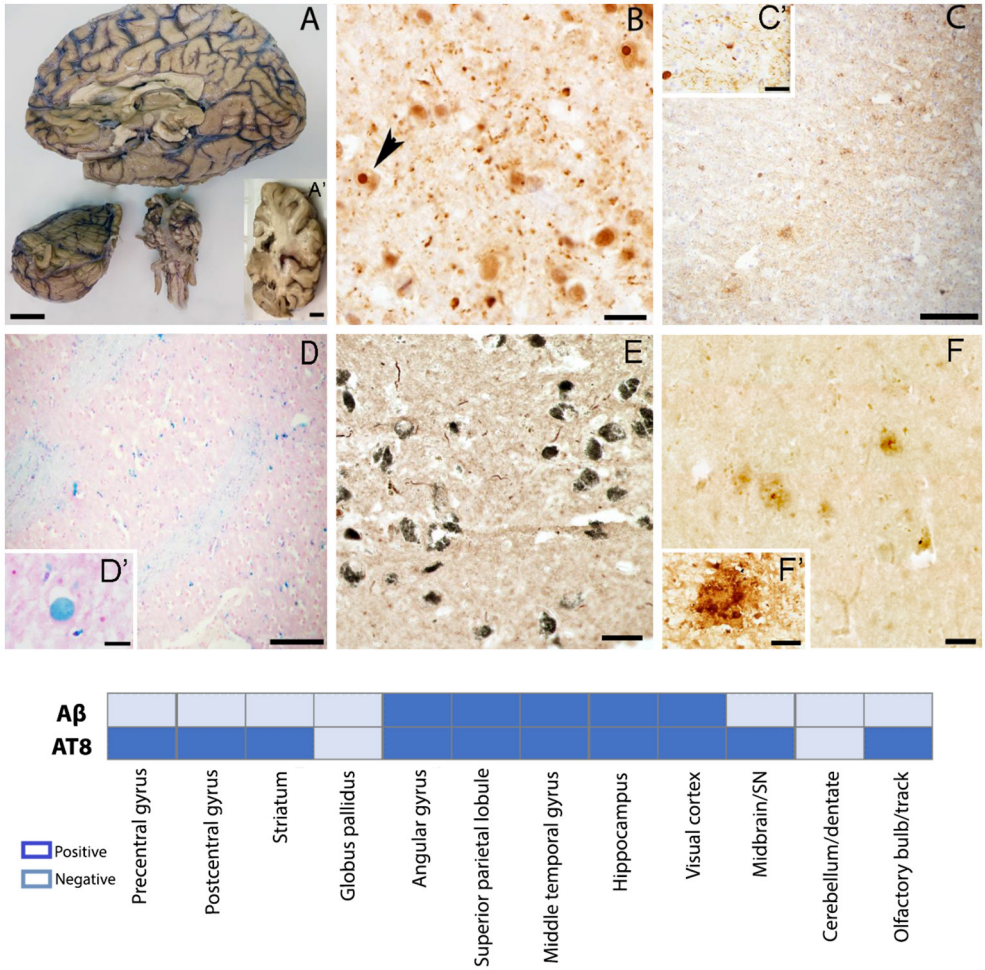

FIGURE 3 | Gross and microscopic brain evaluation. **(A)** Severe frontal and temporal lobes atrophy, thin corpus callosum, sclerotic changes in basilar artery. **(B)** Ubiquitin positive intranuclear inclusions in the post central gyrus confirming definite diagnosis of FXTAS. **(C)** Tau positive plaques and neuropils in the prefrontal gyrus. **(D)** Iron deposits in the globus pallidus. **(E)** Tau positive neuropils in the SN **(F)** Amyloid β positive plaques in the middle temporal gyrus. **(A–A')** scale bar 2 cm; **(B)** scale bar 50 μm; **(C,D)** scale bar 200 μm; **(C')** scale bar 10 μm;; (**D** insert) scale bar 20 μm; **(E)** scale bar 50 μm; **(F)** scale bar 50 μm; **(F')** scale bar 20 μm, Graph shows a summary of brain regions sampled for histopathology evaluation. Aβ, amyloid beta; AT8, phosphorylated Tau.

We found ubiquitin-positive intranuclear inclusions in neurons and astrocytes of the prefrontal cortex (**Figure 3B**), confirming the definite diagnosis of FXTAS. We also noted either neurofibrillary tangles and/or neuropils and/or neuritic plaques in all the studied areas except for the globus pallidus and the cerebellum (**Figure 3C**). Using Braak scoring (Braak and Braak, 1995) for tau pathology a Braak stage V–VI suggests the co-occurrence of Alzheimer-type pathology. High amounts of iron deposits were visualized in the globus pallidus and the putamen consistent with MRI findings (**Figure 3D**). CMBs were noticed in the precentral gyrus, middle temporal gyrus, visual cortex, and brainstem. Tau aggregates were found in the neuromelanin containing neurons of the SN; however, no Lewy bodies were identified by α-synuclein immunostaining (**Figure 3E**), excluding a pathological diagnosis of concomitant Lewy body disease based on current criteria. Extracellular deposition of β-amyloid (core/diffuse plaques) was found in cortical areas and the hippocampus (**Figure 3F**), but absent in the basal ganglia, brainstem, and cerebellum. β-amyloid assessment met Thal phase (2) criteria (Thal et al., 2002); scattered β-amyloid positivity in leptomeningeal and cortical vessels, and capillaries, was also seen, but insufficient to fully assess CAA.

Patient Perspective

Informed consent approved by the University of California Davis IRB committee was obtained at the time of clinical evaluation, for the use of neurocognitive and genetic testing for research purposes (IRB#254134). Brain donation was approved under IRB#215292. The final manuscript was provided for review and acceptance by the family for scientific publication.

DISCUSSION

Both, FXTAS and AD are neurodegenerative diseases that share overlapping cognitive deterioration that includes deficits in information processing speed, working memory and executive function (Grigsby, 2008) and both are known to have a slow progression (Hagerman and Hagerman, 2016, Vermunt et al., 2019) with preclinical phases. A prior postmortem brain study in females with the *FMR1* premutation reported concomitant FXTAS and AD (Tassone et al., 2012). In a small cohort of premutation carriers with and without FXTAS, at least one *APOE ε4* allele was found in 32% of those with FXTAS and it is hypothesized to serve as a genetic risk factor for developing FXTAS (Silva et al., 2013). However, the prevalence of coexistent FXTAS-AD pathology remains unknown. Now, we present a case of rapid motor and cognitive decline. By the time of his first evaluation in our clinic 5 years after his onset of memory and motor deficits, he met clinical criteria for probable Major Neurocognitive Disorder and used a mobility aid. His cognitive and motor abilities continued to decline steeply until death 4 years later.

Neuropsychiatric assessment plays a pivotal role in the early identification of cognitive impairment and neurological degeneration, especially in the prodromal and subsequent phases of AD (Zec, 1993). His neuropsychiatric tests showed reduced executive function abilities including a decrease across all WAIS-IV domains, especially working memory and verbal comprehension even at baseline. His MMSE score of 15, qualified for moderate dementia and further indicated significant impairment in cognitive function, which has been identified as a predictor of AD-associated mortality (Larson et al., 2004). Taken together, his symptoms supported the clinical diagnosis and expected progression of AD from mild to moderate memory issues within 1 year.

The patient's first scan presented with common findings of FXTAS including an MCP sign, thin corpus callosum and WMH (Jacquemont et al., 2003) but his cognitive deficits were strikingly severe for an initial FXTAS visit. A decrease in total brain and cortical volume, along with a significant decrease in temporal structure volume, all found to be related to atrophy in AD (Double et al., 1996), were identified in the scans of *in vivo* brain structures in addition to postmortem imaging and tissue examination. The increase of WMH and the ventricular size was also remarkable. Additionally, postmortem imaging revealed small focal increases in R2* signals, consistent with CMBs in the cerebral white matter consistent with the pathological findings in multiple cortical regions and the SN. Postmortem MRI also revealed increased R2* signals in the deep nuclei such as the putamen and the globus pallidus which presented with high amounts of hemosiderin positive deposits (**Figures 1A–J**). The brain atrophy in this case is faster than what is typically reported with FXTAS, and we hypothesize that those with FXTAS and coexistent Alzheimer-type pathology or perhaps the influence of the *APOE* ε4 alleles may contribute to a faster clinical decline compared to those with FXTAS alone. A prior report by Aydin et al., of a patient with FXTAS and faster than expected cognitive decline of the Alzheimer-type also demonstrated atrophy of the temporal lobes, however, the patient did not have an *APOE* ε4, FXTAS symptoms developed after age 70 and cognitive decline became apparent 10-years after the initial FXTAS diagnosis (Aydin et al., 2020). Both cases share a history of depression. Additional risk factors such as the use of anti-coagulants and vascular disease may have facilitated the progression of brain degeneration and FXTAS symptoms in this case. Such additional factors could have further exacerbated the progression of cognitive decline than just FXTAS and AD alone.

There are two remarkable histopathology findings to be highlighted in this case. First, the absence of Lewy bodies in the neuromelanin-containing neurons of the SN was unexpected since the patient presented mixed tremor in association with bradykinesia and masked facies (**Figure 3E**). However, FXTAS often presents with classic Parkinsonism in the absence of Parkinson's Disease (Salcedo-Arellano et al., 2020). Second, the contrasting burden between Aβ and tau proteins. While the studied brain regions had very few and in some sampled regions absence of amyloid deposits and scattered CAA, tau aggregates were found widely (**Figures 3C,F**) except for the globus pallidus and cerebellum (see graph in **Figure 3** for additional information), a neuropathologic finding that has been observed in about 2–10% of cases in large autopsy studies and recognized as primary age-related tauopathy (PART) (Crary et al., 2014). We do not discard this alternative diagnosis in this case; however, the severe cognitive impairment in multiple domains and the inability of the patient to perform independent activities of daily living is more in accordance with our initial hypothesis of FXTAS-AD. Findings from the postmortem evaluation suggest the coexistence of FXTAS-AD, however, a definite pathological diagnosis will require further evaluation, including CERAD scoring, which was omitted due to differences in tissue thickness requirement.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Univeristy of California Davis IRB. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

MS-A: conception, organization, and execution of the research project (brain dissection, staining, microscopic analysis, and

imaging). Writing of the first draft of the manuscript. DS: execution of the research project (tissue preparation, staining and imaging). Writing of the first draft of the manuscript. JW: execution of the research project (MRI analysis). Writing of the first draft of the manuscript. YM: execution of the research project (study coordinator, MRI acquisition, tissue preparation, staining, microscopic analysis, and imaging). Writing of the first draft of the manuscript. CC: writing of the first draft of the manuscript. PJ: execution of the research project (tissue preparation). Writing of the first draft of the manuscript. AS: execution of the research project (neuropsychiatric assessment). Writing of the first draft of the manuscript. FT: execution of the research project (genetic evaluation). Writing of the first draft of the manuscript. RH and VM-C: conception, organization, and execution of the research project. Review and critique of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

Aydin, E. Y., Schneider, A., Protic, D., Wang, J. Y., Martinez-Cerdeno, V., Tassone, F., et al. (2020). Rapidly progressing neurocognitive disorder in a male with FXTAS and Alzheimer's Disease. *Clin. Interv. Aging* 15, 285–292. doi: 10.2147/CIA.S240314

Braak, H., and Braak, E. (1995). Staging of alzheimer's disease-related neurofibrillary changes. *Neurobiol. Aging* 16, 271–278. doi: 10.1016/0197-4580(95)00021-6

Coupe, P., Catheline, G., Lanuza, E., Manjon, J. V., and Alzheimer's Disease Neuroimaging, I. (2017). Towards a unified analysis of brain maturation and aging across the entire lifespan: a MRI analysis. *Hum. Brain Mapp.* 38, 5501–5518. doi: 10.1002/hbm.23743

Crary, J. F., Trojanowski, J. Q., Schneider, J. A., Abisambra, J. F., Abner, E. L., Alafuzoff, I., et al. (2014). Primary age-related tauopathy (PART): a common pathology associated with human aging. *Acta Neuropathol.* 128, 755–766. doi: 10.1007/s00401-014-1349-0

Double, K. L., Halliday, G. M., Krill, J. J., Harasty, J. A., Cullen, K., Brooks, W. S., et al. (1996). Topography of brain atrophy during normal aging and alzheimer's disease. *Neurobiol. Aging* 17, 513–521. doi: 10.1016/0197-4580(96)00005-X

Filipovic-Sadic, S., Sah, S., Chen, L., Krosting, J., Sekinger, E., Zhang, W., et al. (2010). A novel FMR1 PCR method for the routine detection of low abundance expanded alleles and full mutations in fragile X syndrome. *Clin. Chem.* 56, 399–408. doi: 10.1373/clinchem.2009.136101

Folstein, M. F., and Folstein, S. E., McHugh PRJJopr. (1975). "Mini-mental state": a practical method for grading the cognitive state of patients for the *Clinician* 12, 189–198. doi: 10.1016/0022-3956(75)90026-6

Grigsby, J., Brega, A. G., Engle, K., Leehey, M. A., Hagerman, R. J, Tassone, F., et al. (2008). Cognitive profile of fragile X premutation carriers with and without fragile X-associated tremor/ataxia syndrome. *Neuropsychology* 22, 48–60. doi: 10.1037/0894-4105.22.1.48

Grigsby, J., and Kaye, K. J. C. B. (1996). Behavioral dyscontrol scale: manual ward. *Percept. Mot. Skills* 74, 883–892. doi: 10.2466/pms.1992.74.3.883

Hagerman, R. J., and Hagerman, P. (2016). Fragile X-associated tremor/ataxia syndrome—features, mechanisms and management. *Nat. Rev. Neurol.* 12, 403–412. doi: 10.1038/nrneurol.2016.82

Jacquemont, S., Hagerman, R. J., Leehey, M., Grigsby, J., Zhang, L., Brunberg, J. A., et al. (2003). Fragile X premutation tremor/ataxia syndrome: molecular, clinical, and neuroimaging correlates. *Am. J. Hum. Genet.* 72, 869–878. doi: 10.1086/374321

Larson, E. B., Shadlen, M.-F., Wang, L., McCormick, W. C., Bowen, J. D., Teri, L., et al. (2004). Survival after initial diagnosis of Alzheimer Disease. *Ann. Intern. Med.* 140, 501–509. doi: 10.7326/0003-4819-140-7-200404060-00008

Ligsay, A., El-Deeb, M., Salcedo-Arellano, M. J., Schloemerkemper, N., Grayson, J. S., and Hagerman, R. (2019). General anesthetic use in fragile X spectrum disorders. *J. Neurosurg. Anesthesiol.* 31, 285–290. doi: 10.1097/ANA.0000000000000508

Manjon, J. V., and Coupe, P. (2016). volBrain: an online mri brain volumetry system. *Front. Neuroinform.* 10:30. doi: 10.3389/fninf.2016.00030

Manjon, J. V., Eskildsen, S. F., Coupe, P., Romero, J. E., Collins, D. L., and Robles, M. (2014). Nonlocal intracranial cavity extraction. *Int. J. Biomed. Imaging* 2014:820205. doi: 10.1155/2014/820205

Martinez-Cerdeno, V., Lechpammer, M., Lott, A., Schneider, A., and Hagerman, R. (2015). Fragile X-associated tremor/Ataxia syndrome in a man in His 30s. *JAMA Neurol.* 72, 1070–1073. doi: 10.1001/jamaneurol.2015.1138

Meguro, R., Asano, Y., Odagiri, S., Li, C., Iwatsuki, H., and Shoumura, K. (2007). Nonheme-iron histochemistry for light and electron microscopy: a historical, theoretical and technical review. *Arch. Histol. Cytol.* 70, 1–19. doi: 10.1679/aohc.70.1

Mirra, S. S., Heyman, A., McKeel, D., Sumi, S. M., Crain, B. J., Brownlee, L. M., et al. (1991). The Consortium to establish a registry for Alzheimer's disease (CERAD). Part II. Standardization of the neuropathologic assessment of Alzheimer's disease. *Neurology* 41, 479–486. doi: 10.1212/WNL.41.4.479

Montine, T. J., Creighton, H. P.,Thomas G. B., Eileen, H. B., Nigel, J. C., Dennis, et al. (2012). National Institute on Aging-Alzheimer's Association guidelines for the neuropathologic assessment of Alzheimer's disease: a practical approach. *Acta Neuropathol.* 123, 1–11. doi: 10.1007/s00401-011-0910-3

Muzar, Z., Adams, P. E., Schneider, A., Hagerman, R. J., and Lozano, R. (2014). Addictive substances may induce a rapid neurological deterioration in fragile X-associated tremor ataxia syndrome: a report of two cases. *Intract. Rare Dis. Res.* 3, 162–165. doi: 10.5582/irdr.2014.01023

Muzar, Z., Lozano, R., Schneider, A., Adams, P. E., Faradz, S. M., Tassone, F., et al. (2015). Methadone use in a male with the FMRI premutation and FXTAS. *Am. J. Med. Genet. A.* 167, 1354–1359. doi: 10.1002/ajmg.a.37030

Peran, P., Hagberg, G., Luccichenti, G., Cherubini, A., Brainovich, V., Celsis, P., et al. (2007). Voxel-based analysis of R2* maps in the healthy human brain. *J. Magn. Reson. Imaging* 26, 1413–1420. doi: 10.1002/jmri.21204

Romero, J. E., Coupe, P., Giraud, R., Ta, V. T., Fonov, V., Park, M. T. M., et al. (2017). CERES: A new cerebellum lobule segmentation method. *Neuroimage* 147, 916–924. doi: 10.1016/j.neuroimage.2016.11.003

Salcedo-Arellano, M. J., Wang, J. Y., McLennan, Y. A., Doan, M., Cabal-Herrera, A. M., Jimenez, S., et al. (2021). Cerebral Microbleeds in Fragile X-Associated Tremor/Ataxia Syndrome. *Mov Disord.* doi: 10.1002/mds.28559

Salcedo-Arellano, M. J., Wolf-Ochoa, M. W., Hong, T., Amina, S., Tassone, F., Lechpammer, M., et al. (2020). Parkinsonism versus concomitant Parkinson's

disease in Fragile X-associated Tremor/Ataxia syndrome. *Mov. Disord. Clin. Pract.* 7, 413–418. doi: 10.1002/mdc3.12942

Saldarriaga, W., Salcedo-Arellano, M. J., Rodriguez-Guerrero, T., Rios, M., Fandino-Losada, A., Ramirez-Cheyne, J., et al. (2019). Increased severity of fragile X spectrum disorders in the agricultural community of Ricaurte, Colombia. *Int. J. Dev. Neurosci.* 72, 1–5. doi: 10.1016/j.ijdevneu.2018.10.002

Schmidt, P., Gaser, C., Arsic, M., Buck, D., Förschler, A., Berthele, A., et al. (2012). An automated tool for detection of FLAIR-hyperintense white-matter lesions in Multiple Sclerosis. *Neuroimage* 59, 3774–3783. doi: 10.1016/j.neuroimage.2011.11.032

Seritan, A. L., Kim, K., Benjamin, I., Seritan, I., and Hagerman, R. J. (2016). Risk Factors for Cognitive impairment in Fragile X-associated Tremor/Ataxia syndrome. *J. Geriatr. Psychiatry Neurol.* 29, 328–337. doi: 10.1177/0891988716666379

Silva, F., Rodriguez-Revenga, L., Madrigal, I., Alvarez-Mora, M. I., Oliva, R., and Mila, M. (2013). High apolipoprotein E4 allele frequency in FXTAS patients. *Genet. Med.* 15, 639–642. doi: 10.1038/gim.2013.12

Tassone, F., Greco, C. M., Hunsaker, M. R., Seritan, A. L., Berman, R. F., Gane, L. W., et al. (2012). Neuropathological, clinical and molecular pathology in female fragile X premutation carriers with and without FXTAS. *Genes Brain. Behav.* 11, 577–585. doi: 10.1111/j.1601-183X.2012.00779.x

Tassone, F., Hagerman, R. J., Taylor, A. K., Gane, L. W., Godfrey, T. E., and Hagerman, P. J. (2000). Elevated levels of FMR1 mRNA in carrier males: a new mechanism of involvement in the fragile-X syndrome. *Am. J. Hum. Genet.* 66, 6–15. doi: 10.1086/302720

Tassone, F., Pan, R., Amiri, K., Taylor, A. K., and Hagerman, P. J. (2008). A rapid polymerase chain reaction-based screening method for identification of all expanded alleles of the fragile X (FMR1) gene in newborn and high-risk populations. *J. Mol. Diagn.* 10, 43–49. doi: 10.2353/jmoldx.2008.070073

Thal, D. R., Rüb, U., Orantes, M., and Braak, H. (2002). Phases of Aβ-deposition in the human brain and its relevance for the development of AD. *Neurology* 58:1791. doi: 10.1212/WNL.58.12.1791

Vermunt, L., Sietske, A. M., Ardo, H., Ron H., Isabelle B., Wiesje, M. F., et al. (2019). Duration of preclinical, prodromal, and dementia stages of Alzheimer's disease in relation to age, sex, and APOE genotype. *Alzheim. Dement. J. Alzheim. Assoc.* 15, 888–898. doi: 10.1016/j.jalz.2019.04.001

Wechsler, D. (2008). *WAIS-IV technical and interpretive manual*. Pearson.

Wechsler, D. (2009). *WMS-IV technical and interpretive manual*. Pearson

Weis, S., Kimbacher, M., Wenger, E., and Neuhold, A. (1993). Morphometric analysis of the corpus callosum using MR: correlation of measurements with aging in healthy individuals. *AJNR Am. J. Neuroradiol.* 14:637–645.

Zec, R. F. (1993). "Neuropsychological functioning in Alzheimer's disease," in *Neuropsychology of Alzheimer's Disease and other Dementias*. New York, NY: Oxford University Press, 3–80.

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mGluR5 Negative Modulators for Fragile X: Treatment Resistance and Persistence

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Fragile X syndrome (FXS) is caused by silencing of the human *FMR1* gene and is the leading monogenic cause of intellectual disability and autism. Abundant preclinical data indicated that negative allosteric modulators (NAMs) of metabotropic glutamate receptor 5 (mGluR5) might be efficacious in treating FXS in humans. Initial attempts to translate these findings in clinical trials have failed, but these failures provide the opportunity for new discoveries that will improve future trials. The emergence of acquired treatment resistance ("tolerance") after chronic administration of mGluR5 NAMs is a potential factor in the lack of success. Here we confirm that FXS model mice display acquired treatment resistance after chronic treatment with the mGluR5 NAM CTEP in three assays commonly examined in the mouse model of FXS: (1) audiogenic seizure susceptibility, (2) sensory cortex hyperexcitability, and (3) hippocampal protein synthesis. Cross-tolerance experiments suggest that the mechanism of treatment resistance likely occurs at signaling nodes downstream of glycogen synthase kinase 3α (GSK3α), but upstream of protein synthesis. The rapid emergence of tolerance to CTEP begs the question of how previous studies showed an improvement in inhibitory avoidance (IA) cognitive performance after chronic treatment. We show here that this observation was likely explained by timely inhibition of mGluR5 during a critical period, as brief CTEP treatment in juvenile mice is sufficient to provide a persistent improvement of IA behavior measured many weeks later. These data will be important to consider when designing future fragile X clinical trials using compounds that target the mGluR5-to-protein synthesis signaling cascade.

Keywords: fragile X syndrome, FMRP, glycogen synthase kinase, acquired treatment resistance, drug tolerance, autism, intellectual disability, mGluR5

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INTRODUCTION

Fragile X syndrome (FXS) is the most common monogenic intellectual disability disorder, affecting ∼1:4000 males and 1:8000 females (1). 60–75% of boys and 20–40% of girls with FXS are diagnosed with autism spectrum disorder (ASD), making FXS the most prevalent monogenic cause of ASD (2). In almost all cases, FXS arises from a CGG trinucleotide repeat expansion in the 5' untranslated region of the fragile X mental retardation gene (*FMR1*) which fully silences expression of its protein product, fragile X mental retardation protein (FMRP) (3). Decades of preclinical research

have identified myriad disruptions to brain function in genetically engineered animal models of FXS, greatly advancing our understanding of the underlying molecular and cellular disease mechanisms (4).

Preclinical research has identified two broad classes of pathophysiological mechanisms related to two well-described neuronal functions of FMRP: (1) altered protein synthesis regulation caused by loss of FMRP binding to mRNA (5–13), and (2) altered ion channel function caused by the disruption of FMRP protein-protein interactions with ion channels (9, 14–19). These two disease mechanisms impair synapse development, alter the balance between synaptic excitation and inhibition, and cause widespread increases in neuronal activity (20). To make matters more complicated, the hyperexcitability caused by these proximal molecular defects can feed back to further alter protein synthesis, ion channel function, and synapse development. Short of gene therapy, strategies to tamp down neuronal hyperexcitability and altered proteostasis offer the best prospects to improve the course of FXS, particularly if initiated early in development.

The sheer diversity of ion channels involved in FXS pathology likely limits the potential benefit of targeting any single channel type (21). On the other hand, the myriad consequences of altered protein synthesis regulation in FXS suggested that targeting this process has the potential to confer broad phenotypic improvement. This line of reasoning provided the rationale behind the "mGluR theory of fragile X," as metabotropic glutamate receptor 5 (mGluR5) is widely expressed in the forebrain and known to modulate neuronal protein synthesis (22, 23). Indeed, extensive studies in multiple animal models of FXS have shown that diverse disease phenotypes are corrected by inhibiting mGluR5 or key signaling nodes downstream of this receptor (8, 24, 25). In addition to altered protein synthesis, these phenotypes include but are not restricted to hyperexcitability in sensory neocortex, increased cortical dendritic spine density, epileptiform activity in the hippocampus, audiogenic seizures (AGS), and impaired cognition measured by performance in an inhibitory avoidance (IA) task.

These encouraging animal studies led to human clinical trials using negative allosteric modulators (NAMs) of mGluR5 (mavoglurant and basimglurant). However, these studies failed to demonstrate improvements in the primary therapeutic endpoints (26). The reasons for these failures have been extensively discussed and may include inadequate measures of target engagement and treatment response, suboptimal selection of drug doses and treatment duration, and the older age of the subjects (8, 27, 28). There is also anecdotal evidence that although benefits were observed initially, the effectiveness of treatment faded with chronic dosing (https://www.fraxa.org/fragile-x-clinical-trials-mglur-theory/).

Resolving the obvious disconnect between the robust and highly reproducible rescue achieved in fragile X animal models and the failure to observe efficacy in humans is of enormous importance. Here we focus on one potential explanation for this discrepancy; the development of acquired treatment resistance ("tolerance") that emerges during chronic drug treatment, a common obstacle for neuropsychiatric drug therapy (29). Our study builds on previous observations of diminished mGluR5 NAM effectiveness with repeat dosing in the audiogenic seizure assay (30). In that early work, the protection from AGS conferred by a single *in vivo* injection of the mGluR5 NAM MPEP was significantly reduced (but not eliminated) after repeated daily dosing. This effect appears to be exacerbated when MPEP is combined with a GABA-B receptor agonist (31) and after dosing with CTEP, another mGluR5 NAM with greater selectivity and a much longer half-life (32). In the current study we set out to address three key questions that emerge from these findings. (1) Is acquired treatment resistance to chronic mGluR5 NAM treatment observed in other FXS phenotypic assays? (2) Does resistance to treatment with an mGluR5 NAM impact the effectiveness of an intervention further downstream in the signaling pathway? (3) What accounts for the observation that in assays such as inhibitory avoidance, months long treatment with mGluR5 NAMs corrected behavior?

Our data show acquired treatment resistance following chronic treatment with the mGluR5 NAM CTEP occurs not only in AGS but also in assays of visual cortical hyperexcitability and elevated basal hippocampal protein synthesis. Treatment resistance is not due to increased mGluR5 expression or sensitivity as it cannot be overcome by additional treatment with structurally distinct mGluR5 NAMs. Rather, the mechanism of acquired treatment resistance appears to be downstream of the Ras-ERK1/2 pathway that links mGluR5 activation with increased synaptic protein synthesis. A selective inhibitor of GSK3α was unable to overcome acquired treatment resistance, but a protein synthesis inhibitor was still effective, positioning the mechanism of acquired treatment resistance between GSK3α and translation initiation. Finally, our data reveal that correction of IA deficits in adult mice does not require chronic dosing at all, but rather can be achieved by brief but timely treatment earlier in postnatal development.

Taken together these results provide additional evidence that acquired treatment resistance occurs following chronic mGluR5 inhibition and broadly impacts the ability of these drugs to correct pathophysiological phenotypes. However, treatment resistance is neither inevitable nor an impediment to phenotypic improvements if the pharmacological interventions are timed to occur during critical developmental windows. These findings will be important to consider during study design for future clinical trials in FXS testing compounds that target mGluR5 and the downstream signaling cascade linked to altered proteostasis, especially for patients with demonstrated deficits in protein synthesis (5).

MATERIALS AND METHODS

Study Design

Male *Fmr1* knockout (Fmr1-KO) and wildtype (WT) littermates on the C57BL/6J background were studied in all experiments. The breeding scheme was female Fmr1 heterozygous mice (Jackson Laboratory Stock Number: 003025) crossed with wildtype male C57BL/6J mice (Jackson Laboratory Stock Number: 000664). Sample size was determined by a power analysis or laboratory historical experience and no outliers were

removed from any data sets. Age-matched littermates were randomized to treatment groups and a balanced number of Fmr1-KO and WT mice were used. Mice were group housed on static racks and maintained on a 12:12 h light:dark cycle. The Committee on Animal Care at MIT approved all experimental techniques, and all animals were handled in accordance with NIH and MIT guidelines.

Reagents

The mGluR5 specific negative allosteric modulator CTEP (chloro-4-((2,5-dimethyl-1-(4-(trifluoromethoxy)phenyl)-1H-imidazol-4-yl)ethynyl)pyridine) was purchased from Sigma-Aldrich (SML2306) and administered at 2 mg/kg concentration by intraperitoneal (i.p.) injection. CTEP was prepared daily as a microsuspension in vehicle (0.9% NaCl, 0.3% Tween-80). The GSK3α selective inhibitor BRD0705 was synthesized at the Broad Institute at MIT and confirmed to be of ≥95% purity based on HPLC LC-MS and 1H NMR analysis and administered at 30 mg/kg by i.p. injection. BRD0705 was prepared daily from frozen 50 mM stocks in DMSO. The mGluR5 specific negative allosteric modulator MPEP hydrochloride (2-Methyl-6-(phenylethynyl)pyridine hydrochloride) was purchased from Tocris (1212) and administered to brain slices at 30 μM by bath perfusion. MPEP was prepared daily in vehicle (100% aCSF, see below). The mGluR5 specific negative allosteric modulator MTEP (3-((2-Methyl-1,3-thiazol-4-yl)ethynyl)pyridine hydrochloride) was purchased from Tocris (2921) and administered to brain slices at 1 μM by bath perfusion. MTEP was prepared daily in vehicle (100% aCSF, see below). The translation elongation inhibitor cycloheximide (3-[2-(3,5-Dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide; CHX) was purchased from Sigma-Aldrich (C7698) and administered at 120 mg/kg concentration by i.p. injection and to brain slices at 60 μM by bath perfusion. For i.p. injection CHX was prepared daily in vehicle (0.9% NaCl). For bath application CHX was prepared daily in vehicle (aCSF, see below).

Audiogenic Seizure Assay

AGS experiments were performed as previously described (32). Fmr1-KO and WT mice were housed on static racks to prevent auditory desensitization that occurs with chronic exposure to the ambient noise of ventilated racks. For acute dosing experiments, mice received i.p. injections with vehicle or drug in a separate room 1–2 h prior to exposure to the alarm in a separate room. For chronic CTEP dosing experiments, mice received 3 i.p. CTEP or vehicle injections, one every 48 h, with the final dose occurring 1–2 h prior to testing. All injections began at P23-25 (immediately following weaning). Animals were habituated to the behavioral chamber (28 × 17.5 × 12 cm transparent plastic box) for 1 min prior to stimulus onset. The auditory stimulus was a 125 dB at 0.25 m siren (modified personal alarm, RadioShack model 49-1010, powered from a DC converter). Seizures were scored for incidence during a 2-min stimulus presentation or until the animal reached an AGS endpoint. Wild running/jumping, status epilepticus, respiratory arrest or death were all scored as seizure activity.

Spontaneous Spiking in Visual Cortex

Visual cortical excitability experiments were performed as previously described (32). 350 μm thick acute brain slices containing primary visual cortex were isolated from P20-P21 Fmr1-KO and WT littermate animals or from Fmr1-KO animals that had received chronic or acute CTEP or vehicle injections beginning at P16-P17. Slice were prepared using a Leica Vibratome in ice-cold cutting solution containing (in mM): 87 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 0.5 $CaCl_2$, 7 $MgCl_2$, 20 glucose, 1.3 ascorbate, 75 sucrose, saturated with 95% O_2 and 5% CO_2. Slices were recovered for 30 min at 32°C and then for an additional 2.5 h at room temperature in a modified aCSF containing (in mM): 124 NaCl, 3.5 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 glucose, 0.8 $MgCl_2$, and 1 $CaCl_2$, saturated with 95% O_2 and 5% CO_2. During recordings, slices received a single 0.2 ms duration electrical stimulation of the white matter (clustered bipolar tungsten, FHC) every 30 s using a stimulus intensity between 40 and 80 μA and neuronal activity was recorded by placing a glass recording electrode (∼1 MΩ resistance when filled with aCSF) in layer 5 of primary visual cortex. Extracellular recordings were first collected in vehicle conditions for 30 min (60 trials total), followed by 30 additional minutes in the presence of either 30 μM MPEP or 60 μM cycloheximide. All recordings were acquired with a Multiclamp 700B amplifier (Molecular Devices), amplified 1000 times, filtered between 300 Hz and 10 kHz, and digitized at 25 kHz. Spontaneous spiking events were classified as those occurring between 3.2 s and 30 s after stimulation.

Metabolic Labeling

Metabolic labeling of new protein synthesis was performed as previously described (33). Male P28-P32 Fmr1-KO and WT littermate mice received three CTEP or vehicle i.p. injections over 5 days. One to two hours following the final injection, mice were anesthetized with isoflurane and the hippocampus was rapidly dissected into ice-cold aCSF (in mM: 124 NaCl, 3 KCl, 1.25 NaH_2PO_4, 26 $NaHCO_3$, 10 dextrose, 1 $MgCl_2$, 2 $CaCl_2$, saturated with 95% O_2 and 5% CO_2). Five hundred μm thick hippocampal slices were prepared using a Stoelting Tissue Slicer and transferred into 32.5°C aCSF (saturated with 95% O_2 and 5% CO_2) within 5 min. Slices were incubated in aCSF undisturbed for 3 h to allow recovery of basal protein synthesis and then transferred to either aCSF containing vehicle (dH_2O) or MTEP (1 μM), which was present for the remainder of the experiment. Actinomycin D (25 μM) was added to the chamber for 30 min to inhibit transcription after which slices were transferred to fresh aCSF containing ∼10 mCi/ml [^{35}S] Met/Cys (Perkin Elmer) for an additional 30 min. Slices were then homogenized, and labeled proteins isolated by TCA precipitation. Radiolabel incorporation was measured with a scintillation counter and samples were subjected to a protein concentration assay (Bio-Rad). Data was analyzed as counts per minute per microgram of protein, normalized to the [^{35}S] Met/Cys aCSF used for incubation. The average incorporation of all samples was analyzed and then normalized to percent wildtype for each experiment.

Inhibitory Avoidance Assay

Inhibitory avoidance (IA) experiments were performed as previously described (32). Group housed male Fmr1-KO and WT littermate mice received three injections of 2 mg/kg CTEP or vehicle over 5 days beginning at ∼P28. Drug was then withheld for ∼4 weeks. Two days prior to IA testing, all animals were habituated to handling which consisted of scruffing mice for ∼10 s to verify the ear tag number, followed by 5 min of resting in the gloved hands of the investigator and being allowed to freely explore while the tail was lightly restrained to prevent escape. On the day of testing, ∼P60 animals were placed into the dark compartment of the IA training box (a two-chambered Perspex box consisting of a lighted safe side and a dark shock side separated by a trap door) for 30 seconds followed by 90 s in the light compartment for habituation. Following the habituation period, the door separating the two compartments was opened and animals were allowed to enter the dark compartment. Latency to enter following door opening was recorded (0-h time point, collected between 8 and 10 a.m.); 1 animal with baseline entrance latency of >120 s. was excluded. After each animal stepped completely into the dark compartment with all four paws, the sliding door was closed and the animal received a single scrambled foot-shock (0.5 mA, 2.0 s) via electrified steel rods in the floor of the box. This foot shock intensity and duration caused each animal to vocalize and jump. Animals then remained in the dark compartment for 15–30 s following the shock and were then placed in a fresh cage. After all members of a single cage experienced the training, mice were returned as a group to their home cage. Six to seven hours following IA training, mice received a retention test (6-h time point, collected between 2 and 4 p.m.). During post-acquisition retention testing, each animal was placed in the lit compartment as in training; after a 90 s delay, the door opened, and the latency to enter the dark compartment was recorded (cut-off time 537 s). The order of animals run was preserved between trials. For inhibitory avoidance extinction (IAE) training, animals were allowed to explore the dark compartment of the box for 200 s in the absence of foot-shock (animals remaining in the lit compartment after the cutoff were gently guided, using an index card, into the dark compartment); following IAE training, animals were returned to their home cages. Twenty-four hours following initial IA training, mice received a second retention test (24-h time point, collected between 8 and 10 a.m.). Animals were tested in the same way as at the 6-h time point, followed by a second 200 s extinction trial in the dark side of the box; following training, animals were again returned to their home cages. Forty-eight hours following avoidance training, mice received a third and final retention test (48-h time point, collected between 8 and 10 a.m.).

Statistical Analysis

All experiments were performed by trained experimenters blind to genotype and drug treatment, and included same day, interleaved controls for genotype and drug treatment. All data are expressed as mean ±SEM, with n values represented in the figures and figure legends. Unless indicated otherwise, the n values stated in figures and figure legends represent numbers of animals [in experiments in which more than one measurement was taken from an animal (metabolic labeling and visual cortical spiking activity), the value representing this animal is the average of technical replicates]. Differences in audiogenic seizure incidence were determined using a two-tailed Fisher's exact test. For brain slice electrophysiology experiments, the effect of genotype or drug treatment of brain slices was determined using a paired two-tailed Student's *t*-test. For protein synthesis in **Figures 4B**,**C**, differences between genotype and drug treatment were determined using a two-way ANOVA with Šídák's multiple comparisons test for *post-hoc* analysis. For protein synthesis in **Figure 4D**, differences between genotype and drug stimulation conditions were determined using a three-way repeated measures ANOVA and Šídák's multiple comparisons test for *post-hoc* analysis. Differences between genotypes and treatment in the inhibitory avoidance assay were determined using a repeated measures two-way ANOVA with Greenhouse-Geisser correction and Tukey's post-test for *post-hoc* analysis.

RESULTS

Chronic mGluR5 Inhibition Induces Treatment Resistance of Audiogenic Seizure Susceptibility Which Is Overcome by Inhibiting Protein Synthesis

The mGluR theory posits that glutamate acting via mGluR5 stimulates protein synthesis that is pathogenic in FXS. Early studies showed that a single dose of MPEP was sufficient to suppress audiogenic seizures in the Fmr1-KO mouse (30). This result was later confirmed using CTEP, a NAM with increased selectivity for mGluR5 (34), as well as with several other structurally distinct mGluR5 inhibitors (35). However, as initially suggested by Yan et al. (30), we showed in a recent study that treatment resistance develops rapidly, with as few as 3 doses of CTEP over 5 days [**Figure 1**, data reproduced from (32)].

It is usually assumed that the effects of mGluR5 NAMs on fragile X phenotypes are due to suppression of excessive protein synthesis that occurs in the absence of FMRP. However, some fragile X phenotypes that respond to these NAMs are expressly not improved by inhibiting protein synthesis directly with cycloheximide (CHX) or other mRNA translation inhibitors (36–39). Thus, to make sense of the effects of chronic CTEP, it was important to determine if the AGS phenotype was a readout of excessive ongoing protein synthesis. To that end, we administered CHX (120 mg/kg) via a single i.p. injection in Fmr1-KO animals 1.5 h prior to a 2-min exposure to the 120 dB auditory stimulus. The AGS phenotype was faithfully recapitulated in vehicle-treated animals and corrected by CHX, confirming that in Fmr1-KO mice the expression of AGS susceptibility is indeed protein synthesis dependent (**Figures 2A,B**).

We next wondered if development of resistance to CTEP would also render CHX ineffective, in which case the reemergence of the AGS phenotype after chronic treatment would be explained by an entirely different pathogenic mechanism. To address this question, Fmr1-KO mice were

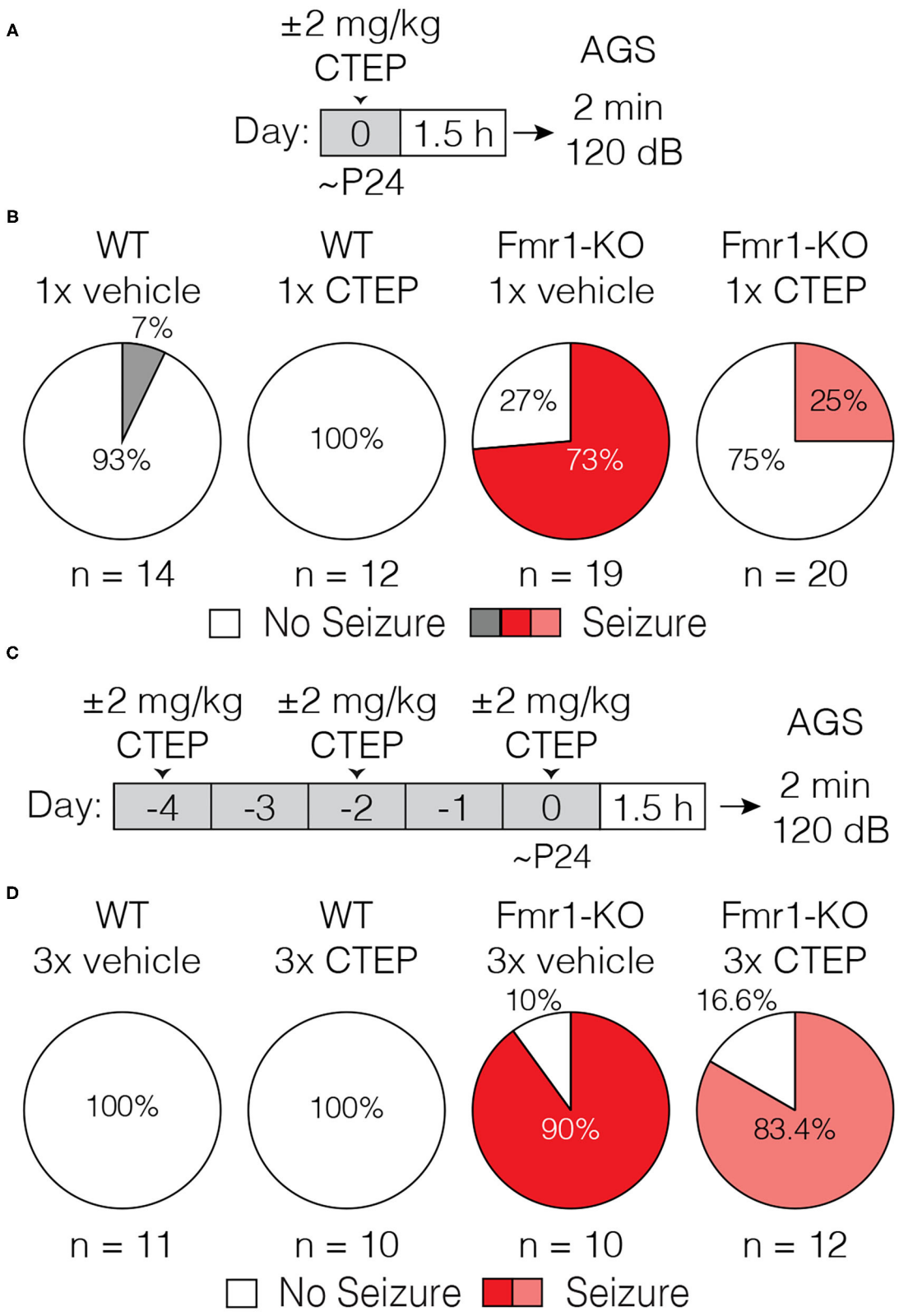

FIGURE 1 | Acute but not chronic administration of the mGluR5 NAM CTEP ameliorates audiogenic seizures in Fmr1-KO mice. **(A)** Schematic shows acute CTEP dose schedule and AGS experimental design. **(B)** Fmr1-KO mice treated with vehicle exhibit increased susceptibility to audiogenic seizures compared to WT treated with vehicle (two-tailed Fisher's exact test: $p = 0.0001$) and an acute dose of 2 mg/kg CTEP significantly reduced AGS incidence in Fmr1-KO mice (two-tailed Fisher's

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FIGURE 1 | exact test: $p = 0.0038$). **(C)** Schematic shows chronic CTEP dose schedule and AGS experimental design. **(D)** Chronic treatment (3 doses over 5 days) with 2 mg/kg CTEP no longer alleviates susceptibility to audiogenic seizures, indicating the development of acquired treatment resistance (Two-tailed Fisher's exact test: Fmr1-KO vehicle treated vs. CTEP treated: p = 1.0). Data re-plotted from (32).

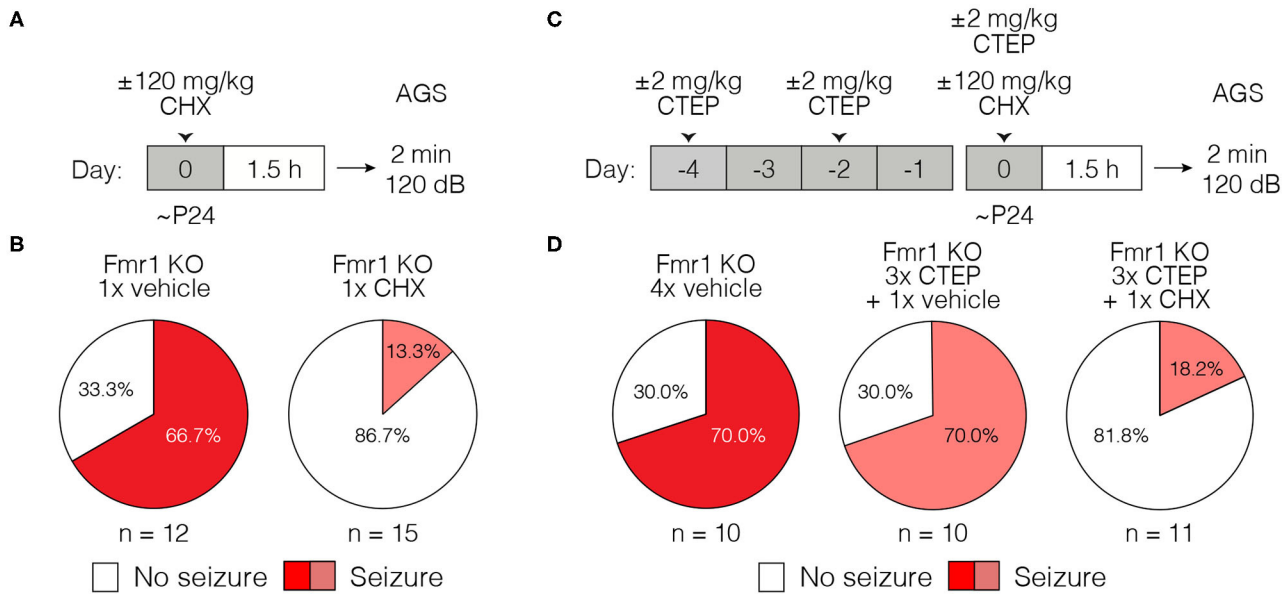

FIGURE 2 | Acute cycloheximide treatment ameliorates audiogenic seizures in Fmr1-KO mice and overcomes acquired CTEP resistance. **(A)** Schematic shows acute CHX dose schedule and AGS experimental design. **(B)** Acute treatment with 120 mg/kg CHX significantly reduced AGS incidence in Fmr1-KO mice (Two-tailed Fisher's exact test: $p = 0.0069$). **(C)** Schematic shows acute cycloheximide after chronic CTEP dose schedule and AGS experimental design. **(D)** Chronic CTEP (3 doses of 2 mg/kg over 5 days) causes treatment resistance that can be overcome by an acute injection of 120 mg/kg CHX immediately prior to assessing AGS (Two-tailed Fisher's exact test: Fmr1-KO 4x vehicle vs. Fmr1-KO 3x CTEP + CHX: $p = 0.0131$; Fmr1-KO 3x CTEP + vehicle vs. Fmr1-KO 3x CTEP + CHX: $p = 0.0131$).

first injected with vehicle or CTEP (2 mg/kg) 3 times over 5 days to induce treatment resistance. Then, following the third injection, mice were injected with vehicle or CHX and AGS susceptibility was measured. Fmr1-KO mice again demonstrated a robust AGS phenotype that was, as expected, not corrected by CTEP after chronic exposure (**Figures 2C,D**). However, CHX treatment was still able to acutely suppress AGS. Thus, CTEP resistance likely entails upregulation of signaling pathways that converge on protein synthesis regulation.

Acquired Treatment Resistance of Cortical Hyperexcitability

AGS susceptibility is a complex behavioral phenotype that arises from the absence of FMRP in the inferior colliculus (40). To explore the generality of the phenomenon of acquired treatment resistance, we employed an *in vitro* assay of neuronal hyperexcitability in visual cortical slices from Fmr1-KO mice. Previous studies have shown that layer 5 neurons display increased spontaneous spiking activity in the Fmr1-KO that is corrected acutely by treatments targeting signaling downstream of mGluR5 (12, 32). We confirmed this cellular phenotype (**Figures 3A,B**) and then investigated the sensitivity to acute exposure to the mGluR5 NAM MPEP in Fmr1-KO mice following treatment *in vivo* with either vehicle or 3x CTEP. In brain slices prepared from Fmr1-KO animals injected with vehicle, bath application of the mGluR5 inhibitor MPEP (30 μM) rapidly (within 30 min) reduced the number of action potentials (**Figures 3C–E**). If animals were first treated *in vivo* with a single dose of CTEP shortly before brain slice preparation, we also observed a complete suppression of aberrant spiking activity. Inhibition of mGluR5 by CTEP is known to be long-lasting and survive slice preparation, even a day later (34). Therefore, it was not surprising that we observed no additional suppression of spiking by bath applied MPEP in these experiments, presumably because mGluR5 was fully inhibited by the CTEP treatment (**Figures 3D,E**).

The findings were very different after 3 doses of CTEP *in vivo*. Layer 5 neurons displayed the characteristic hyperexcitability phenotype which could no longer be reversed by MPEP. The inability of bath applied MPEP (on top of the residual CTEP in the tissue) to correct the hyperexcitability suggests that the molecular mechanism of acquired treatment resistance is unlikely to be explained simply by an upregulation of receptors in the membrane. However, as was observed for the AGS phenotype, inhibition of protein synthesis with CHX was still effective in correcting this phenotype even after the development of resistance to the mGluR5 NAMs (**Figures 3D,E**).

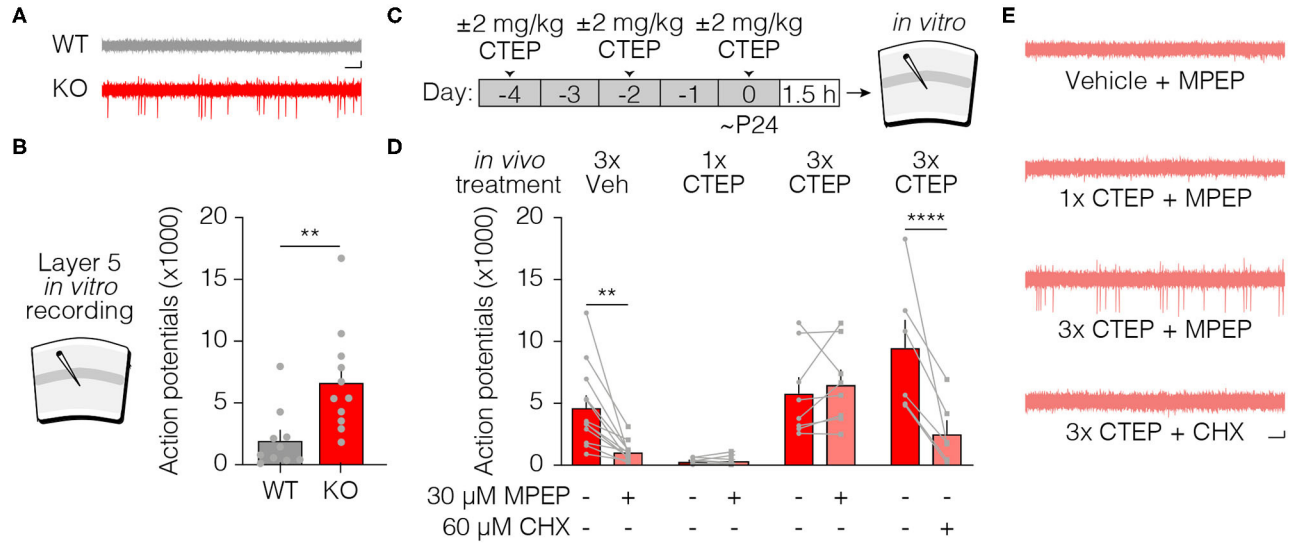

FIGURE 3 | Acute but not chronic CTEP treatment ameliorates hyperexcitability in Fmr1-KO layer 5 of primary visual cortex. **(A)** Representative traces of extracellular recordings in layer 5 of primary visual cortex from wild type (black) and Fmr1-KO (red) animals show increased spontaneous firing in Fmr1-KO slices (Scale bar represents 200 μV by 200 ms). **(B)** Layer 5 neurons in Fmr1-KO mice have significantly increased spontaneous action potentials compared to wild type littermates (paired *t*-test: wild type vs. Fmr1-KO: $p = 0.0062$). **(C)** Schematic shows acute and chronic CTEP dose schedules and visual cortical excitability experimental design. **(D1)** Elevated spontaneous activity in layer 5 primary visual cortical slices from Fmr1-KO mice is significantly reduced by bath application of 30 μM MPEP (Two-tailed paired *t*-test: Fmr1-KO 3x vehicle + vehicle vs. Fmr1-KO 3x vehicle + MPEP: $p = 0.0024$) or **(D2)** an acute injection *in vivo* of 2 mg/kg CTEP (Two-tailed paired *t*-test: Fmr1-KO 1x CTEP + vehicle vs. Fmr1-KO 1x CTEP + MPEP: $p = 0.1693$). **(D3)** Chronic 2 mg/kg CTEP (3 doses over 5 days) leads to treatment resistance of spontaneous activity in layer 5 primary visual cortex that is not overcome by bath application of 30 μM MPEP (Two-tailed paired *t*-test: Fmr1-KO 3x CTEP + vehicle vs. Fmr1-KO 3x CTEP + MPEP: $p = 0.4327$) but **(D4)** is significantly reduced by bath application of 60 μM CHX (Two-tailed paired *t*-test: Fmr1-KO 3x CTEP + vehicle vs. Fmr1-KO 3x CTEP + CHX: $p = 0.0102$). **(E)** Representative traces of extracellular recordings in layer 5 primary visual cortex showing spontaneous activity in Fmr1-KO slices treated with bath applied 30 μM MPEP, animals injected acutely with 2 mg/kg CTEP, and animals injected chronically (3 doses over 5 days) with 2 mg/kg CTEP followed by bath application of either 30 μM MPEP or 60 μM CHX (Scale bar represents 200 μV by 200 ms). Data are displayed as mean ±SEM. $^{**}p < 0.01$, $^{****}p < 0.0001$.

Chronic mGluR5 Inhibition Fails to Normalize Elevated Hippocampal Protein Synthesis Rates in Fmr1-KO Mice

An elevated rate of basal protein synthesis in the hippocampus of Fmr1-KO mice and rats is a hallmark fragile X phenotype, and it is rescued by inhibiting proteins in the signaling cascade linking mGluR5 activation with protein synthesis (5, 12, 32, 33, 41–45). Consistent with these prior studies, we found that a single dose of CTEP *in vivo* corrects the protein synthesis phenotype measured ∼4 h later in hippocampal slices (**Figures 4A,B**). Once again, this effect was lost after dosing CTEP 3 times over 5 days (**Figure 4C**). Thus, the acquired resistance to treatment with the mGluR5 NAM was not restricted to excitability phenotypes, but also generalizes to a core biochemical phenotype in another brain region.

We next asked if supplemental bath application of a second mGluR5 NAM could overcome the resistance observed after repeated dosing with CTEP treatment *in vivo*. In these experiments we used MTEP, which is more selective and potent than MPEP (46, 47). Although MTEP reversed the fragile X phenotype in animals treated previously with vehicle, this effect was lost in animals treated chronically with CTEP (**Figure 4D**). This finding again suggests that this phenomenon is not due to changes in the expression of the mGluR5 receptor but rather depends on an adaption of intracellular signaling.

The Mechanism of Acquired Treatment Resistance Lies Downstream of GSK3α Activation

The signaling pathways that link mGluR5 activation with the regulation of protein synthesis are well-described (27). The Ras-ERK1/2 pathway is of considerable interest in FXS, as inhibitors such as lovastatin and metformin that target this signaling arm correct myriad phenotypes in the Fmr1-KO mouse and rat (**Figure 5A**) (12, 48, 49), as well as biochemical phenotypes in platelets and neurons derived from patients with FXS (43, 50). We therefore could elucidate where in this signaling cascade acquired treatment resistance arises by testing which interventions more proximal to protein synthesis can overcome the effects of chronic CTEP. We recently demonstrated that the paralog-specific GSK3α inhibitor BRD0705 corrects AGS susceptibility, cortical hyperexcitability, and basal protein synthesis phenotypes in Fmr1-KO animals (32). In wildtype mice, BRD0705 inhibits the stimulation of protein synthesis by

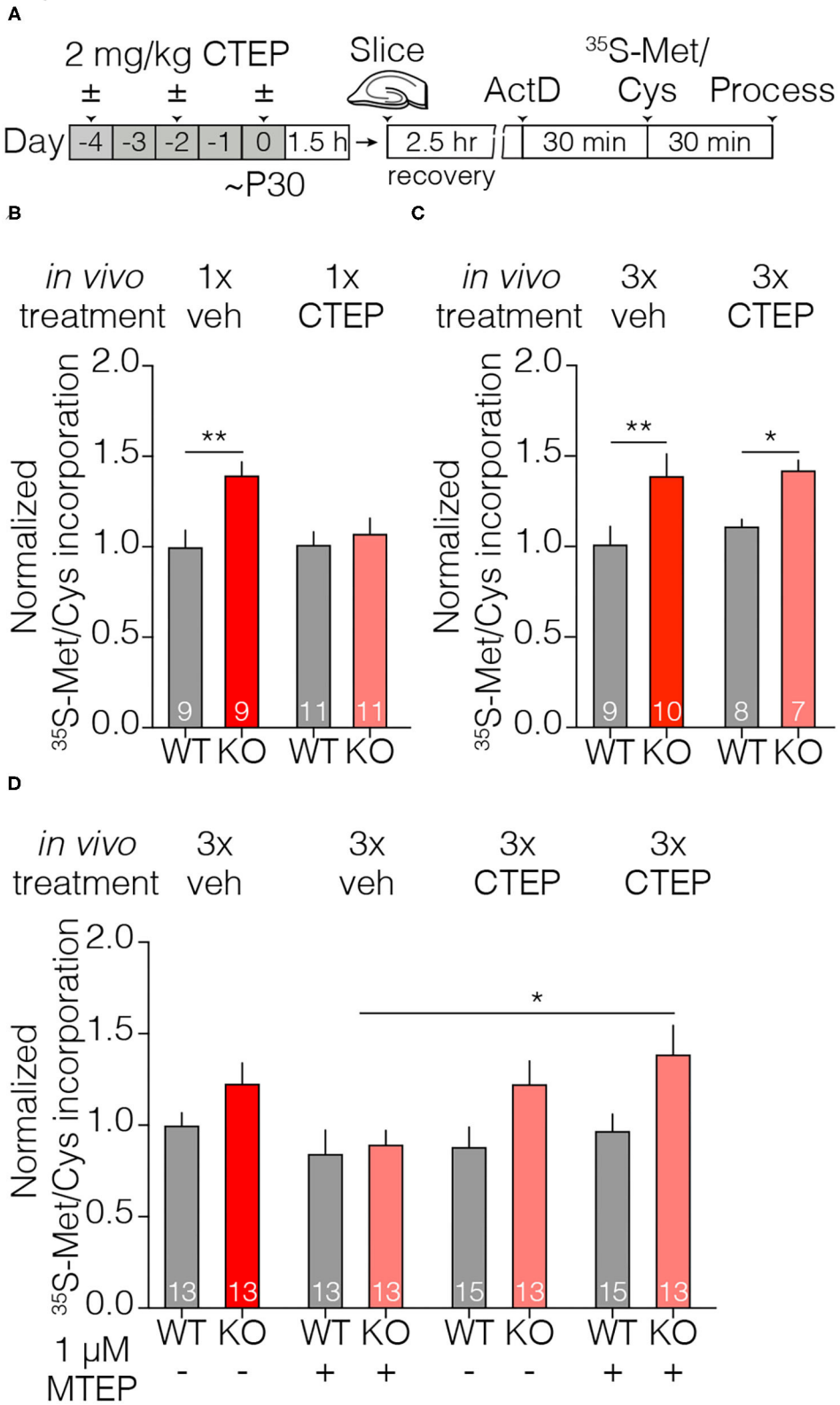

FIGURE 4 | Acute but not chronic CTEP treatment ameliorates elevated basal protein synthesis rates in Fmr1-KO hippocampal slices. **(A)** Schematic shows acute and chronic CTEP dose schedules and hippocampal metabolic labeling experimental design. **(B)** Basal protein synthesis rates are increased in hippocampal slices prepared from Fmr1-KO mice compared to wildtype littermate animals and acute *in vivo* treatment with 2 mg/kg CTEP restores Fmr1-KO protein synthesis rates to wildtype levels. There was a statistically significant effect of genotype [two-way ANOVA, $F_{(1,36)} = 8.341$, $p = 0.0066$] and a significant interaction between genotype

(Continued)

FIGURE 4 | and treatment. [two-way ANOVA, $F_{(1,36)} = 4.501$, $p = 0.0408$; Šídák's multiple comparisons test: wild type 1x vehicle vs. Fmr1-KO 1x vehicle p = 0.0036, wild type 1x CTEP vs. Fmr1-KO 1x CTEP $p = 0.8183$). **(C)** Chronic (3 doses over 5 days) *in vivo* treatment with 2 mg/kg CTEP has no effect on radiolabel incorporation in hippocampal slices from wildtype or Fmr1-KO animals. There was a statistically significant effect of genotype [two-way ANOVA, $F_{(1,30)} = 15.13$, $p = 0.0005$; Šídák's multiple comparisons test: wild type 3x vehicle vs. Fmr1-KO 3x vehicle $p = 0.0082$, wild type 3x CTEP vs. Fmr1-KO 3x CTEP $p = 0.0410$]. **(D)** Bath application of 1 μM MTEP reduces elevated basal protein synthesis rates in Fmr1-KO hippocampal slices to wild type levels but has no effect on hippocampal slices from animals injected with chronic (3 doses over 5 days) 2.0 mg/kg CTEP. There was a statistically significant effect of genotype [three-way ANOVA, $F_{(1,50)} = 8.013$, $p = 0.0067$] and a significant interaction between *in vivo* treatment and *in vitro* treatment. [three-way ANOVA, $F_{(1,50)} = 8.536$, $p = 0.0052$; Šídák's multiple comparisons test: Fmr1-KO 3x vehicle + MTEP vs. Fmr1-KO 3x CTEP + MTEP $p = 0.0347$). Data are displayed as mean ±SEM. $^*p < 0.05$, $^{**}p < 0.01$.

mGluR5 activation, but not phosphorylation of ERK1/2, thus indicating that it acts well downstream in the signaling pathway. We therefore reasoned that by acting more proximal to protein synthesis regulation, BRD0705 might be able to overcome the acquired treatment resistance induced by chronic CTEP.

To test this hypothesis, resistance to treatment with mGluR5 NAMs was first induced by 3x CTEP injections and then the effect of BRD0705 was examined. In controls, receiving only vehicle before BRD0705, we confirmed the suppression of AGS by inhibiting GSK3α. However, previous chronic exposure to CTEP eliminated the ameliorative effect of BRD0705 (**Figures 5B,C**). These findings suggest that whatever cellular adaption is responsible for acquisition of treatment resistance following CTEP treatment, it is likely to occur downstream of signaling by GSK3α (but upstream of protein synthesis; see **Figure 2D**).

Temporary Early Intervention With CTEP Corrects Inhibitory Avoidance Behavior 1 Month After Treatment

We have demonstrated the development of rapid treatment resistance in audiogenic seizure susceptibility, visual cortical hyperexcitability, and hippocampal protein synthesis, after only 3 doses of CTEP. However, a previous study showed that a deficit in inhibitory avoidance (IA), a contextual learning behavior, was corrected in mice receiving doses of CTEP (2 mg/kg p.o.) every other day for >1 month (achieving a steady state receptor occupancy of >80%) (34). Clearly that chronic dosing schedule would have caused treatment resistance in the assays employed here.

How might we reconcile these disparate findings? A recent study using the rat model of fragile X offers a clue (48). They demonstrated that temporary administration of lovastatin for 5 weeks starting at 1 month of age produced a persistent improvement of cognitive behavior that could be measured many weeks after the drug was discontinued. These findings suggest that brief interventions during a postnatal critical period may be sufficient to produce lasting effects. Thus, we wondered if the effect of CTEP on IA memory observed previously by Michalon et al. (34) was actually not a consequence of continuous inhibition of mGluR5, but rather was due to the fortuitous timing of the first dose(s) of CTEP.

To assess this hypothesis, we treated Fmr1-KO mice and wild type littermates with vehicle or CTEP (2 mg/kg i.p.) three times over 5 days beginning at postnatal day 28 and then withheld the drug for 4 weeks and measured inhibitory avoidance behavior beginning at postnatal day 58 (**Figure 6A**). Vehicle injected Fmr1-KO animals replicated the well-described IA deficits in memory acquisition and extinction compared to vehicle treated wild type littermates (**Figure 6B**). However, early and temporary intervention with CTEP fully restored memory acquisition and extinction in Fmr1-KO mice, providing evidence for a critical window in which mGluR5 inhibition produces a durable behavioral improvement. These findings suggest that the long-term treatment regimen deployed in previous studies was unnecessary for the behavioral rescue.

DISCUSSION

Our results confirm the previous observation of acquired resistance to mGluR5 inhibition in the AGS assay (30–32) and demonstrate that this applies to two additional fragile X phenotypes: cortical hyperexcitability and exaggerated protein synthesis. These behavioral, cellular, and biochemical phenotypes arise from dysfunction in three different parts of the brain—inferior colliculus, primary visual cortex, and hippocampus—suggesting that acquired treatment resistance is a general phenomenon, not restricted to one part of the nervous system or one functional readout.

One feature that these fragile X phenotypes have in common, however, is that they have been shown to be corrected rapidly (within tens of minutes) by acute inhibition of mGluR5 signaling in drug naïve animals (12, 30, 33). This rapid drug response has historically been attributed to suppression of mGluR5-regulated protein synthesis. In support of this interpretation, we show here (to our knowledge, for the first time) that both increased susceptibility to AGS and cortical hyperexcitability are also reversed acutely by CHX, an inhibitor of mRNA translation (**Figures 2**, **3**). These findings suggest the existence of pathogenic protein species that are rapidly depleted by inhibiting mGluR5 or protein synthesis. Identifying these protein(s) will be of great interest as they represent potential therapeutic targets that might allow for more precise molecular interventions that do not require manipulating proteostasis directly.

At this point, the mechanism for the acquired treatment resistance is unknown, but our experiments help to narrow the possibilities. We note that use of the term "tolerance" has been avoided in this paper, because it is typically used to describe the reduced effectiveness of receptor *agonists* with prolonged exposure. Desensitization of G-protein coupled receptors upon ligand binding is a well-known phenomenon, and is accounted for by changes in receptor surface expression and/or the

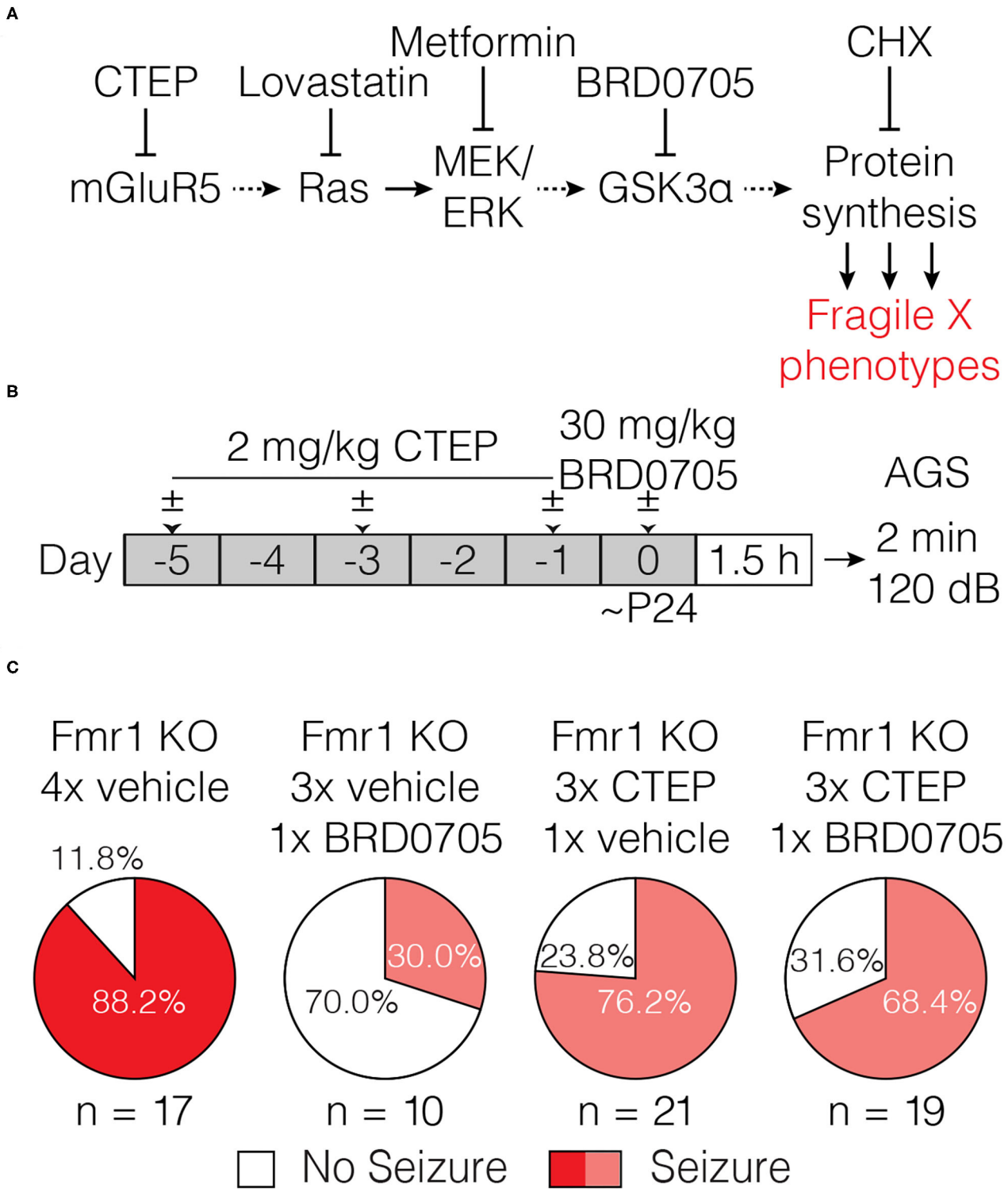

FIGURE 5 | Inhibiting GSK3α does not overcome audiogenic seizure treatment resistance induced by chronic CTEP. **(A)** Some elements of the signaling pathway that couples mGluR5 to protein synthesis. AGS susceptibility in fragile X can be corrected by the protein synthesis inhibitor CHX and by compounds acting at several different nodes in this pathway, including GSK3α. **(B)** Schematic shows drug dosing schedule and AGS experimental design. **(C)** Chronic (3 doses over 5 days) treatment with 2.0 mg/kg CTEP followed by a vehicle injection does not alter Fmr1-KO audiogenic seizure susceptibility (two-tailed Fisher's exact test: Fmr1-KO 4x vehicle vs. Fmr1-KO 3x CTEP +1x vehicle $p = 0.4267$). A single injection of 30 mg/kg BRD0705 normalizes audiogenic seizure susceptibility in Fmr1-KO mice but has no effect on seizure incidence in Fmr1-KO mice treated with chronic (3 doses over 5 days) 2.0 mg/kg CTEP (two-tailed Fisher's exact test: Fmr1-KO 4x vehicle vs. Fmr1-KO 3x vehicle + BRD0705 $p = 0.0036$; two-tailed Fisher's exact test: Fmr1-KO 4x vehicle vs. Fmr1-KO 3x CTEP + BRD0705 $p = 0.2357$).

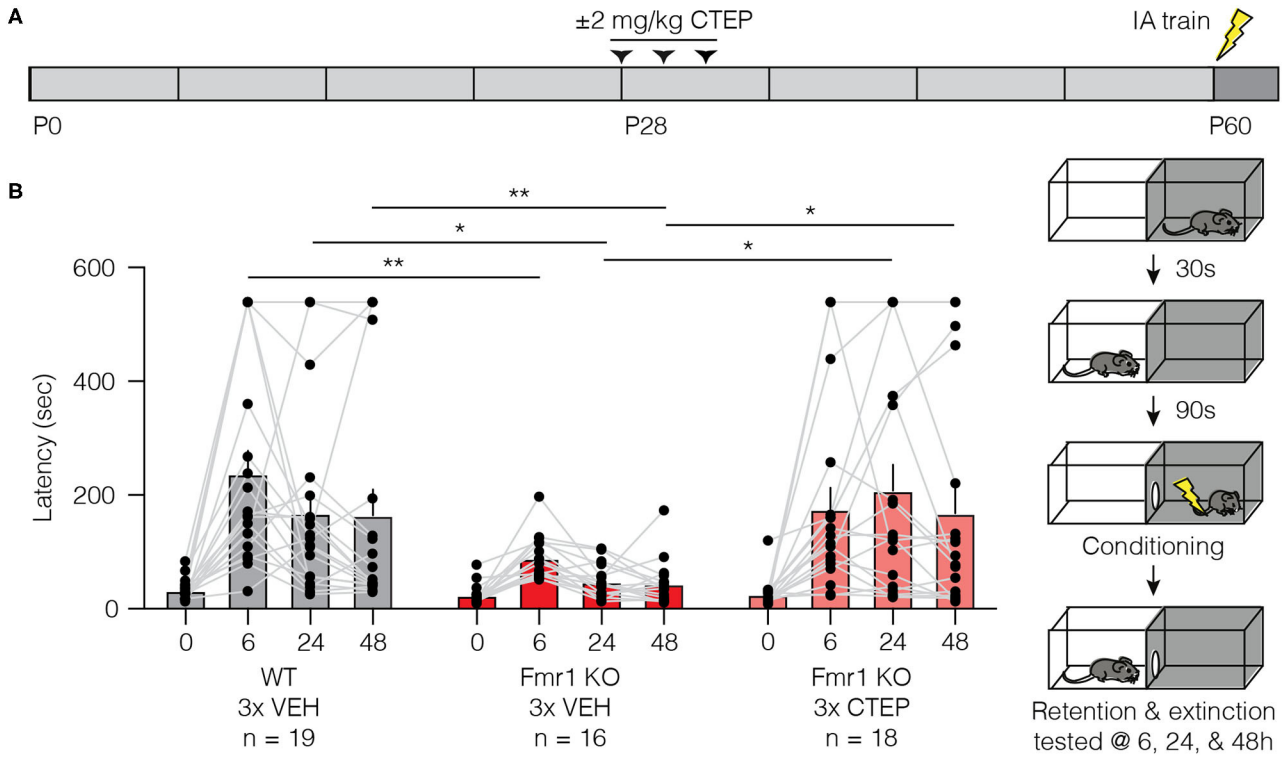

FIGURE 6 | Brief treatment of juvenile Fmr1-KO mice with CTEP normalizes inhibitory avoidance measured 1 month after the end of treatment. **(A)** Schematic shows when during the developmental timeline CTEP is administered and IA experimental design. **(B)** There was a statistically significant interaction between treatment and time point (repeated measures two-way ANOVA with Greenhouse-Geisser correction, $F_{(6,150)} = 2.684$, $p = 0.0064$). Fmr1-KO mice treated with vehicle displayed impaired acquisition and extinction of IA learning compared to vehicle treated wild type mice (wild type vehicle vs. Fmr1-KO vehicle, Tukey's post-test at time 0 h $p = 0.3488$, at 6 h $p = 0.0048$, at 24 h $p = 0.0116$, at 48 h $p = 0.0444$) and CTEP treated Fmr1-KO mice (Fmr1-KO vehicle vs. Fmr1-KO 3x CTEP, Tukey's post-test at time 0 h $p = 0.9756$, at 6 h $p = 0.1024$, at 24 h $p = 0.0067$, at 48 h $p = 0.0416$). Data are displayed as mean ±SEM. $^*p < 0.05$, $^{**}p < 0.01$.

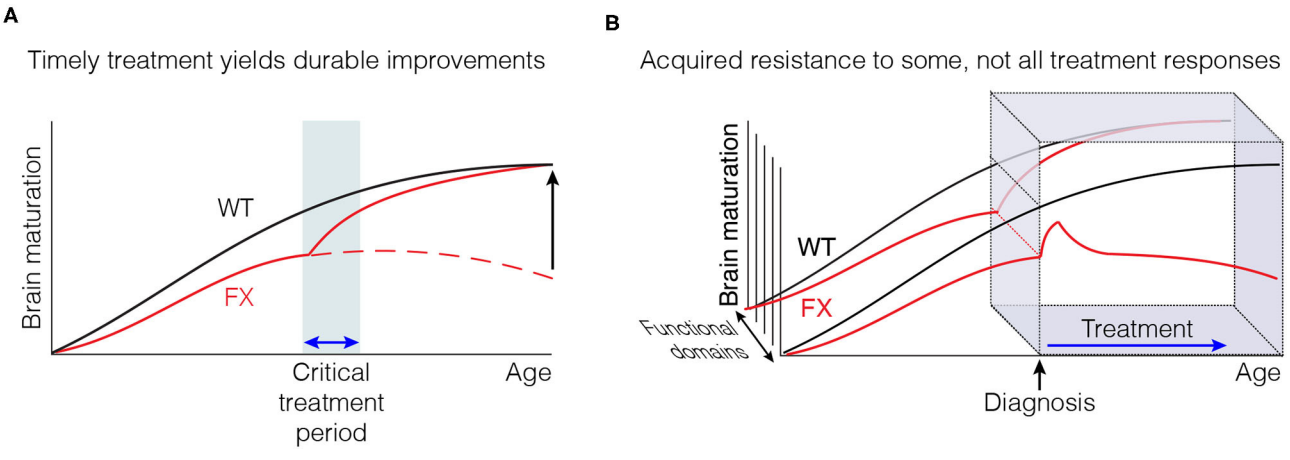

FIGURE 7 | Timely therapeutic interventions have the potential to correct the derailed postnatal development of some cognitive functions. **(A)** Some improvements may not be immediately apparent, but emerge with time after treatment is discontinued. **(B)** Some measures of improvement may not be as susceptible to acquired treatment resistance as others.

coupling of the receptors to their G-proteins (51–53). It is possible that an inverse process—sensitization or increased expression of mGluR5—after chronic NAM treatment accounts for the diminished effectiveness of treatment, but several lines of evidence suggest otherwise. First, in the visual cortex hyperexcitability and hippocampal protein synthesis assays, the lost effectiveness of acute CTEP after chronic dosing was not mitigated by the bath application of another mGluR5 NAM (**Figures 3**, **4**). Considered with the previous finding that mGluR5 protein expression is not increased after development of NAM resistance in the AGS assay (31), these data suggest that the mechanism lies downstream of the receptor. This interpretation is supported by the additional finding that resistance to CTEP blocks the effectiveness of inhibiting GSK3α, an enzyme believed to act downstream of ERK in the pathway coupling mGluR5 to protein synthesis (**Figure 5**) (32). Thus, the treatment resistance we observe here is best conceptualized as an intracellular adaptation to chronic loss of signaling through mGluR5. The re-expression of pathogenic proteins that cause neuronal hyperexcitability after chronic CTEP presumably is the ultimate basis for acquired treatment resistance, because phenotypic rescue is still possible using a protein synthesis inhibitor.

A previous study did not assess the effects of chronic CTEP administration on hippocampal protein synthesis but clearly demonstrated that chronic, weeks long, CTEP dosing fully restored many other phenotypes including inhibitory avoidance, dendritic spine morphology, and overactivity of ERK and mTOR (34). One important clue to resolving this discrepancy comes from our finding that early and temporary modulation of mGluR5 with CTEP rescues inhibitory avoidance behavior measured 4 weeks after withholding CTEP. There now exists an emerging body of evidence that there may be critical developmental windows during which manipulating mGluR5 signaling may restore normal developmental trajectories leading to long lasting behavioral improvements. A recent series of experiments using the Ras/ERK inhibitor lovastatin showed that temporary treatment early in juvenile development of Fmr1-KO rats corrected learning and memory behavioral deficits weeks after the drug had been withheld (48). Importantly, the rat study also demonstrated that hippocampal protein synthesis was rescued weeks after lovastatin was withheld suggesting the same may be observed for CTEP.

Taken together, these data lead to the hypothesis that the sustained correction of some phenotypes observed after chronic CTEP may have been due to the restoration of a normal developmental trajectory early in treatment, rather than reflecting the need for sustained modulation of mGluR5 signaling throughout postnatal maturation (**Figure 7A**). Future studies must continue to refine the timing of these early therapeutic windows and determine if they are specific to individual phenotypes or if they permit the correction of a wide range of fragile X phenotypes. More broadly, these new findings highlight the utility of model systems like the Fmr1-KO mouse and rat to fully examine how the timing of initiation and duration of treatments affects behavioral rescue outcomes.

Recognition of both the risk of acquired treatment resistance, and the opportunity of inducing a persistent improvement with timely intervention during development, should inform the design of future clinical trials. These insights are also usefully applied to the interpretation of the previous trials in FXS using mGluR5 NAMs. The first thing to note is that acquired treatment resistance is not an inevitable consequence of inhibiting mGluR5 signaling. For example, although we saw profound loss of effectiveness in the AGS assay after only 3 doses of CTEP over 5 days, BRD0705 (targeting GSK3α) and MPEP (an mGluR5 NAM) retain anticonvulsant activity after 5-6 daily doses (31, 32). Both compounds have a far shorter half-life than CTEP, and daily dosing does not produce continuous inhibition of mGluR5 signaling. Thus, pulsatile rather than sustained inhibition of mGluR5 might have durable therapeutic efficacy while avoiding maladaptive treatment resistance. We note that there are considerable differences in the pharmacokinetic properties of mGluR5 NAMs that have advanced to human studies, with affinities that range over 200-fold (54). In published FXS trials, mavoglurant (AFQ056), with a half-life of ∼12 h (55), was dosed twice daily for 12 weeks (26). It is entirely possible that the failure of these trials to demonstrate benefit in their primary endpoint was due to acquired treatment resistance. It is also possible that not all efficacy measures are equally affected by treatment resistance (**Figure 7B**), perhaps explaining why significant improvements could still be observed using other functional measures, notably in subjects receiving the lower drug doses (56). Regardless, our findings make the case for limiting the duration of continuous treatment, perhaps by providing rest periods, to prevent development of drug resistance that may have masked some benefits of mGluR5 NAMs in previous clinical trials.

Another factor that clearly needs to be considered is the age at treatment onset. Our findings together with those in the rat model of fragile X (48) suggest that timely postnatal intervention may be sufficient to restore some cognitive functions by correcting the trajectory of subsequent brain circuit development. The youngest age in the mavoglurant clinical trial was 12 years old. It is possible that this age of treatment onset was too late, or that the corrective effect of treatment on brain development takes longer than 12 weeks to be detectable, as has been suggested by observations during the open-label extension period lasting over 2 years (57). Taken together, the evidence indicates that it is premature to abandon the mGluR theory as an organizing principle for developing new disease-modifying therapies in FXS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Committee on Animal Care at MIT, conforming to guidelines from the National

Institutes of Health and the Association for Assessment and Accreditation of Laboratory Animal Care International.

AUTHOR CONTRIBUTIONS

PM, DS, RS, AH, and MB conceived of the experiments. PM conducted and analyzed the hippocampal protein synthesis experiments. PM, RS, and AH conducted and analyzed the AGS experiments. DS conducted and analyzed the inhibitory avoidance experiment. RS conducted and analyzed the extracellular recording experiments. DS, AH, and MB prepared the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Lozano R, Azarang A, Wilaisakditipakorn T, Hagerman RJ. Fragile X syndrome: a review of clinical management. *Intractable Rare Dis Res.* (2016) 5:145–57. doi: 10.5582/irdr.2016.01048
2. Lee M, Martin GE, Berry-Kravis E, Losh M. A developmental, longitudinal investigation of autism phenotypic profiles in fragile X syndrome. *J Neurodev Disord.* (2016) 8:47. doi: 10.1186/s11689-016-9179-0
3. Penagarikano O, Mulle JG, Warren ST. The pathophysiology of fragile x syndrome. *Annu Rev Genomics Hum Genet.* (2007) 8:109–29. doi: 10.1146/annurev.genom.8.080706.092249
4. Telias M. Molecular mechanisms of synaptic dysregulation in fragile X syndrome and autism spectrum disorders. *Front Mol Neurosci.* (2019) 12:51. doi: 10.3389/fnmol.2019.00051
5. Jacquemont S, Pacini L, Jonch AE, Cencelli G, Rozenberg I, He Y, et al. Protein synthesis levels are increased in a subset of individuals with fragile X syndrome. *Hum Mol Genet.* (2018) 27:2039–51. doi: 10.1093/hmg/ddy099
6. Pal R, Bhattacharya A. Modelling protein synthesis as A biomarker in fragile X syndrome patient-derived cells. *Brain Sci.* (2019) 9:59. doi: 10.3390/brainsci9030059
7. Richter JD, Bassell GJ, Klann E. Dysregulation and restoration of translational homeostasis in fragile X syndrome. *Nat Rev Neurosci.* (2015) 16:595–605. doi: 10.1038/nrn4001
8. Stoppel LJ, Osterweil EK, Bear MF. The mGluR theory of fragile X: from mice to men. In: Wilemsen R, Kooy RF, editors. *Fragile X Syndrome*. Academic Press (2017). p. 173–204. doi: 10.1016/B978-0-12-804461-2.00009-3
9. Ronesi JA, Collins KA, Hays SA, Tsai NP, Guo W, Birnbaum SG, et al. Disrupted Homer scaffolds mediate abnormal mGluR5 function in a mouse model of fragile X syndrome. *Nat Neurosci.* (2012) 15:431–40. doi: 10.1038/nn.3033
10. Zhao W, Chuang SC, Bianchi R, Wong RK. Dual regulation of fragile X mental retardation protein by group I metabotropic glutamate receptors controls translation-dependent epileptogenesis in the hippocampus. *J Neurosci.* (2011) 31:725–34. doi: 10.1523/JNEUROSCI.2915-10.2011
11. Zhao W, Chuang SC, Young SR, Bianchi R, Wong RK. Extracellular glutamate exposure facilitates group I mGluR-mediated epileptogenesis in the hippocampus. *J Neurosci.* (2015) 35:308–15. doi: 10.1523/JNEUROSCI.1944-14.2015
12. Osterweil EK, Chuang SC, Chubykin AA, Sidorov M, Bianchi R, Wong RK, et al. Lovastatin corrects excess protein synthesis and prevents epileptogenesis in a mouse model of fragile X syndrome. *Neuron.* (2013) 77:243–50. doi: 10.1016/j.neuron.2012.01.034
13. Chuang SC, Zhao W, Bauchwitz R, Yan Q, Bianchi R, Wong RK. Prolonged epileptiform discharges induced by altered group I metabotropic glutamate receptor-mediated synaptic responses in hippocampal slices of a fragile X mouse model. *J Neurosci.* (2005) 25:8048–55. doi: 10.1523/JNEUROSCI.1777-05.2005
14. Deng PY, Rotman Z, Blundon JA, Cho Y, Cui J, Cavalli V, et al. FMRP regulates neurotransmitter release and synaptic information transmission by modulating action potential duration via BK channels. *Neuron.* (2013) 77:696–711. doi: 10.1016/j.neuron.2012.12.018
15. Zhang Y, Brown MR, Hyland C, Chen Y, Kronengold J, Fleming MR, et al. Regulation of neuronal excitability by interaction of fragile X mental retardation protein with slack potassium channels. *J Neurosci.* (2012) 32:15318–27. doi: 10.1523/JNEUROSCI.2162-12.2012
16. Deng PY, Klyachko VA. Genetic upregulation of BK channel activity normalizes multiple synaptic and circuit defects in a mouse model of fragile X syndrome. *J Physiol.* (2016) 594:83–97. doi: 10.1113/JP271031
17. Deng PY, Carlin D, Oh YM, Myrick LK, Warren ST, Cavalli V, et al. Voltage-independent SK-channel dysfunction causes neuronal hyperexcitability in the hippocampus of Fmr1 knock-out mice. *J Neurosci.* (2019) 39:28–43. doi: 10.1523/JNEUROSCI.1593-18.2018
18. Strumbos JG, Brown MR, Kronengold J, Polley DB, Kaczmarek LK. Fragile X mental retardation protein is required for rapid experience-dependent regulation of the potassium channel Kv3.1b. *J Neurosci.* (2010) 30:10263–71. doi: 10.1523/JNEUROSCI.1125-10.2010
19. Zhang Y, Bonnan A, Bony G, Ferezou I, Pietropaolo S, Ginger M, et al. Dendritic channelopathies contribute to neocortical and sensory hyperexcitability in Fmr1(-/y) mice. *Nat Neurosci.* (2014) 17:1701–9. doi: 10.1038/nn.3864
20. Contractor A, Klyachko VA, Portera-Cailliau C. Altered neuronal and circuit excitability in fragile X syndrome. *Neuron.* (2015) 87:699–715. doi: 10.1016/j.neuron.2015.06.017
21. Deng PY, Klyachko VA. Channelopathies in fragile X syndrome. *Nat Rev Neurosci*. (2021) 22:275–89. doi: 10.1038/s41583-021-00445-9
22. Huber KM, Gallagher SM, Warren ST, Bear MF. Altered synaptic plasticity in a mouse model of fragile X mental retardation. *Proc Natl Acad Sci USA*. (2002) 99:7746–50. doi: 10.1073/pnas.122205699
23. Bear MF, Huber KM, Warren ST. The mGluR theory of fragile X mental retardation. *Trends Neurosci.* (2004) 27:370–7. doi: 10.1016/j.tins.2004.04.009
24. Dolen G, Bear MF. Role for metabotropic glutamate receptor 5 (mGluR5) in the pathogenesis of fragile X syndrome. *J Physiol.* (2008) 586:1503–8. doi: 10.1113/jphysiol.2008.150722
25. Bhakar AL, Dolen G, Bear MF. The pathophysiology of fragile X (and what it teaches us about synapses). *Annu Rev Neurosci.* (2012) 35:417–43. doi: 10.1146/annurev-neuro-060909-153138
26. Berry-Kravis E, Des Portes V, Hagerman R, Jacquemont S, Charles P, Visootsak J, et al. Mavoglurant in fragile X syndrome: results of two randomized, double-blind, placebo-controlled trials. *Sci Transl Med.* (2016) 8:321ra5. doi: 10.1126/scitranslmed.aab4109
27. Berry-Kravis EM, Lindemann L, Jonch AE, Apostol G, Bear MF, Carpenter RL, et al. Drug development for neurodevelopmental disorders: lessons learned from fragile X syndrome. *Nat Rev Drug Discov.* (2018) 17:280–99. doi: 10.1038/nrd.2017.221
28. Howe JR, Bear MF, Golshani P, Klann E, Lipton SA, Mucke L, et al. The mouse as a model for neuropsychiatric drug development. *Curr Biol.* (2018) 28:R909–14. doi: 10.1016/j.cub.2018.07.046

29. McMillan DE. Psychoactive drugs: tolerance sensitization by A. J. Goudie, M. W. Emmett-Oglesby. *Psychopharmacology.* (1991) 103:431–5. doi: 10.1007/BF02244240
30. Yan QJ, Rammal M, Tranfaglia M, Bauchwitz RP. Suppression of two major Fragile X Syndrome mouse model phenotypes by the mGluR5 antagonist MPEP. *Neuropharmacology.* (2005) 49:1053–66. doi: 10.1016/j.neuropharm.2005.06.004
31. Pacey LK, Tharmalingam S, Hampson DR. Subchronic administration and combination metabotropic glutamate and GABAB receptor drug therapy in fragile X syndrome. *J Pharmacol Exp Ther.* (2011) 338:897–905. doi: 10.1124/jpet.111.183327
32. McCamphill PK, Stoppel LJ, Senter RK, Lewis MC, Heynen AJ, Stoppel DC, et al. Selective inhibition of glycogen synthase kinase 3alpha corrects pathophysiology in a mouse model of fragile X syndrome. *Sci Transl Med.* (2020) 12:eaam8572. doi: 10.1126/scitranslmed.aam8572
33. Osterweil EK, Krueger DD, Reinhold K, Bear MF. Hypersensitivity to mGluR5 and ERK1/2 leads to excessive protein synthesis in the hippocampus of a mouse model of fragile X syndrome. *J Neurosci.* (2010) 30:15616–27. doi: 10.1523/JNEUROSCI.3888-10.2010
34. Michalon A, Sidorov M, Ballard TM, Ozmen L, Spooren W, Wettstein JG, et al. Chronic pharmacological mGlu5 inhibition corrects fragile X in adult mice. *Neuron.* (2012) 74:49–56. doi: 10.1016/j.neuron.2012.03.009
35. Westmark PR, Dekundy A, Gravius A, Danysz W, Westmark CJ. Rescue of Fmr1(KO) phenotypes with mGluR5 inhibitors: MRZ-8456 versus AFQ-056. *Neurobiol Dis.* (2018) 119:190–8. doi: 10.1016/j.nbd.2018.08.008
36. Nosyreva ED, Huber KM. Metabotropic receptor-dependent long-term depression persists in the absence of protein synthesis in the mouse model of fragile X syndrome. *J Neurophysiol.* (2006) 95:3291–5. doi: 10.1152/jn.01316.2005
37. Auerbach BD, Bear MF. Loss of the fragile X mental retardation protein decouples metabotropic glutamate receptor dependent priming of long-term potentiation from protein synthesis. *J Neurophysiol.* (2010) 104:1047–51. doi: 10.1152/jn.00449.2010
38. Hays SA, Huber KM, Gibson JR. Altered neocortical rhythmic activity states in Fmr1 KO mice are due to enhanced mGluR5 signaling and involve changes in excitatory circuitry. *J Neurosci.* (2011) 31:14223–34. doi: 10.1523/JNEUROSCI.3157-11.2011
39. Thomazeau A, Bosch M, Essayan-Perez S, Barnes SA, De Jesus-Cortes H, Bear MF. Dissociation of functional and structural plasticity of dendritic spines during NMDAR and mGluR-dependent long-term synaptic depression in wild-type and fragile X model mice. *Mol Psychiatry.* (2020). doi: 10.1038/s41380-020-0821-6 Available online at: https://www.nature.com/articles/s41380-020-0821-6
40. Gonzalez D, Tomasek M, Hays S, Sridhar V, Ammanuel S, Chang CW, et al. Audiogenic seizures in the Fmr1 knock-out mouse are induced by Fmr1 deletion in subcortical, VGlut2-expressing excitatory neurons and require deletion in the inferior colliculus. *J Neurosci.* (2019) 39:9852–63. doi: 10.1523/JNEUROSCI.0886-19.2019
41. Dolen G, Osterweil E, Rao BS, Smith GB, Auerbach BD, Chattarji S, et al. Correction of fragile X syndrome in mice. *Neuron.* (2007) 56:955–62. doi: 10.1016/j.neuron.2007.12.001
42. Stoppel LJ, Auerbach BD, Senter RK, Preza AR, Lefkowitz RJ, Bear MF. Beta-Arrestin2 couples metabotropic glutamate receptor 5 to neuronal protein synthesis and is a potential target to treat fragile X. *Cell Rep.* (2017) 18:2807–14. doi: 10.1016/j.celrep.2017.02.075
43. Utami KH, Yusof N, Kwa JE, Peteri UK, Castren ML, Pouladi MA. Elevated de novo protein synthesis in FMRP-deficient human neurons and its correction by metformin treatment. *Mol Autism.* (2020) 11:41. doi: 10.1186/s13229-020-00350-5
44. Gkogkas CG, Khoutorsky A, Cao R, Jafarnejad SM, Prager-Khoutorsky M, Giannakas N, et al. Pharmacogenetic inhibition of eIF4E-dependent Mmp9 mRNA translation reverses fragile X syndrome-like phenotypes. *Cell Rep.* (2014) 9:1742–55. doi: 10.1016/j.celrep.2014.10.064
45. Qin M, Kang J, Smith CB. Increased rates of cerebral glucose metabolism in a mouse model of fragile X mental retardation. *Proc Natl Acad Sci USA.* (2002) 99:15758–63. doi: 10.1073/pnas.242377399
46. Anderson JJ, Rao SP, Rowe B, Giracello DR, Holtz G, Chapman DF, et al. [3H]Methoxymethyl-3-[(2-methyl-1,3-thiazol-4-yl)ethynyl]pyridine binding to metabotropic glutamate receptor subtype 5 in rodent brain: *in vitro* and *in vivo* characterization. *J Pharmacol Exp Ther.* (2002) 303:1044–51. doi: 10.1124/jpet.102.040618
47. Cosford ND, Tehrani L, Roppe J, Schweiger E, Smith ND, Anderson J, et al. 3-[(2-Methyl-1,3-thiazol-4-yl)ethynyl]-pyridine: a potent and highly selective metabotropic glutamate subtype 5 receptor antagonist with anxiolytic activity. *J Med Chem.* (2003) 46:204–6. doi: 10.1021/jm025570j
48. Asiminas A, Jackson AD, Louros SR, Till SM, Spano T, Dando O, et al. Sustained correction of associative learning deficits after brief, early treatment in a rat model of Fragile X Syndrome. *Sci Transl Med.* (2019) 11:aao0498. doi: 10.1126/scitranslmed.aao0498
49. Gantois I, Khoutorsky A, Popic J, Aguilar-Valles A, Freemantle E, Cao R, et al. Metformin ameliorates core deficits in a mouse model of fragile X syndrome. *Nat Med.* (2017) 23:674–7. doi: 10.1038/nm.4335
50. Pellerin D, Caku A, Fradet M, Bouvier P, Dube J, Corbin F. Lovastatin corrects ERK pathway hyperactivation in fragile X syndrome: potential of platelet's signaling cascades as new outcome measures in clinical trials. *Biomarkers.* (2016) 21:497–508. doi: 10.3109/1354750X.2016.1160289
51. Abreu N, Acosta-Ruiz A, Xiang G, Levitz J. Mechanisms of differential desensitization of metabotropic glutamate receptors. *Cell Rep.* (2021) 35:109050. doi: 10.1016/j.celrep.2021.109050
52. Scheefhals N, Catsburg LAE, Westerveld ML, Blanpied TA, Hoogenraad CC, MacGillavry HD. Shank proteins couple the endocytic zone to the postsynaptic density to control trafficking and signaling of metabotropic glutamate receptor 5. *Cell Rep.* (2019) 29:258–69 e8. doi: 10.1016/j.celrep.2019.08.102
53. Dhami GK, Ferguson SS. Regulation of metabotropic glutamate receptor signaling, desensitization and endocytosis. *Pharmacol Therap.* (2006) 111:260–71. doi: 10.1016/j.pharmthera.2005.01.008
54. Bennett KA, Sergeev E, MacSweeney CP, Bakker G, Cooper AE. Understanding exposure-receptor occupancy relationships for metabotropic glutamate receptor 5 negative allosteric modulators across a range of preclinical and clinical studies. *J Pharmacol Exp Ther.* (2021) 377:157–68. doi: 10.1124/jpet.120.000371
55. Walles M, Wolf T, Jin Y, Ritzau M, Leuthold LA, Krauser J, et al. Metabolism and disposition of the metabotropic glutamate receptor 5 antagonist (mGluR5) mavoglurant (AFQ056) in healthy subjects. *Drug Metab Dispos.* (2013) 41:1626–41. doi: 10.1124/dmd.112.050716
56. Hessl D, Harvey D, Sansone S, Crestodina C, Chin J, Joshi R, et al. Effects of mavoglurant on visual attention and pupil reactivity while viewing photographs of faces in Fragile X syndrome. *PLoS ONE.* (2019) 14:e0209984. doi: 10.1371/journal.pone.0209984
57. Hagerman R, Jacquemont S, Berry-Kravis E, Des Portes V, Stanfield A, Koumaras B, et al. Mavoglurant in Fragile X syndrome: results of two open-label, extension trials in adults and adolescents. *Sci Rep.* (2018) 8:16970. doi: 10.1038/s41598-018-34978-4

Conflict of Interest: MB is a founder of Allos Pharma, developing treatments for fragile X.

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REVIEW
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Heterogeneity in Fragile X Syndrome Highlights the Need for Precision Medicine-Based Treatments

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Fragile X syndrome (FXS) is the most frequent monogenic cause of autism or intellectual disability, and research on its pathogenetic mechanisms has provided important insights on this neurodevelopmental condition. Nevertheless, after 30 years of intense research, efforts to develop treatments have been mostly unsuccessful. The aim of this review is to compile evidence from existing research pointing to clinical, genetic, and therapeutic response heterogeneity in FXS and highlight the need of implementing precision medicine-based treatments. We comment on the high genetic and phenotypic heterogeneity present in FXS, as a contributing factor to the difficulties found during drug development. Given that several clinical trials have showed a non-negligeable fraction of positive responders to drugs targeting core FXS symptoms, we propose that success of clinical trials can be achieved by tackling the underlying heterogeneity in FXS by accurately stratifying patients into drug-responder subpopulations. These precision medicine-based approaches, which can be first applied to well-defined monogenic diseases such as FXS, can also serve to define drug responder profiles based on specific biomarkers or phenotypic features that can associate patients with different genetic backgrounds to a same candidate drug, thus repositioning a same drug for a larger number of patients with NDDs.

Keywords: Fragile X syndrome, precision medicine-based treatments, autism spectrum disorders, biomarkers, monogenic disease, heterogeneity

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INTRODUCTION: HETEROGENEITY BEYOND IDIOPATHIC NEURODEVELOPMENTAL DISORDERS

Neurodevelopmental disorders (NDDs) are a group of prevalent and highly heterogeneous conditions characterized by impairment in "personal, social, academic, or occupational functioning" with onset early in development, which include autism spectrum disorder (ASD), intellectual disability (ID), attention deficit hyperactivity disorder (ADHD), communication disorders, specific learning disorders, and motor disorders (1); moreover, the definition can also include some neuropsychiatric disorders such as schizophrenia and bipolar disorder, and other neurologic disorders such as cerebral palsy or epilepsy (2). Comorbidity of two or more of these disorders is observed at rates higher than what it would be expected by chance, suggesting the

existence of clusters of shared biological mechanisms (3, 4). While cooccurrence of neurological features and conditions is frequently observed, very often, NDDs also include a variety of extra-neurological clinical signs such as hypotonia, dysmorphology, cardiologic or metabolic features, as well as gastrological problems such as constipation or diarrhea, which are specially frequent in ASD and ADHD (5–7), or immunological abnormalities (8, 9). The recent advances in genotyping and sequencing technologies have propelled the identification of risk/causal genes, which has pointed to remarkable genetic heterogeneity among and within specific NDDs; for intellectual disability alone, pathogenic mutations in more than 1,000 genes have been confirmed in the SysID database (a systematic and manually curated catalog of ID-associated genes) (10). While syndromic neurodevelopmental conditions associated to mutations in one single gene are by definition genetically more homogeneous, they still involve varying degrees of phenotype and genetic heterogeneity (11). Further complexifying matters: well-defined clinical entities can be caused by mutations in several genes (as it is the case for Noonan syndrome, linked to mutations in 14 different genes) (12), while mutations occurring in a same gene can result into a wide spectrum of symptoms, as exemplified by *MECP2* mutations in Rett syndrome (13). Adjunctly, different kinds of mutations can result in different pathophysiological mechanisms, as recently exemplified by mutations in *SATB1*, in which three different kinds of variants were associated to distinct pathological consequences (14).

Fragile X syndrome (FXS) is a well-characterized NDD syndrome, caused by deficiency of FMRP (Fragile X Mental Retardation Protein, encoded by *FMR1*), an RNA-binding protein that regulates editing, translation, stability, and transport of a large number of neuronal mRNAs (15). FXS has been extensively studied in the last 30 years. It was first clinically described in 1943 as a form of X-linked inherited intellectual disability (16). In 1969, Lubs developed the chromosomal test for FXS (17), although the causal gene, *FMR1* (Fragile X Mental Retardation 1), and the mutational basis of FXS, were not discovered until 1991 (18). FXS is classically caused by an expansion of >200 CGG repeats in the promoter region of *FMR1* (hereinafter referred as full *FMR1* mutation); this leads to the promoter methylation and transcriptional silencing of *FMR1*. Nowadays, FXS represents the most frequently identified monogenic cause of ASD and inherited intellectual disability (19). But despite being a genetically well-characterized syndrome, there is considerable heterogeneity within patients with FXS, and the condition remains a high unmet medical need. This supports the necessity for deeper characterization of the population with FXS to enable the development of efficient treatments.

CLINICAL AND GENETIC HETEROGENEITY IN FXS

A considerably high level of clinical and genetic heterogeneity can be found in FXS. Despite being a highly recognizable syndrome from the clinical point of view, patients with FXS manifest a wide spectrum of behavioral phenotypes, although some of them such as attention/hyperactivity, hyperarousal, anxiety, and aggression episodes are commonly seen. Females with FXS show a similar spectrum of behavioral difficulties compared to males with FXS, but with milder symptoms (20). Notably, there is a strong association between FXS and autism spectrum disorder (ASD), as ∼50% of FXS male and 20% female patients meet DSM-5 criteria for this disorder, and FXS is the leading monogenic cause of ASD (21). Although autistic features are not present in all individuals it is highly speculated that FXS and some groups of patients with idiopathic ASD present with shared pathophysiology, as defects in many proteins that interact with FMRP have also been associated with idiopathic ASD (15). Importantly, greater severity and lower level of functioning is associated with ASD co-morbidity in FXS (22–24). A higher prevalence of seizures, sleep problems, and co-occurring problematic behaviors, especially aggressive/disruptive behavior, is found in the pediatric population with FXS and a diagnosis of ASD than in the FXS population without ASD (19). Individuals with FXS also widely differ in level of cognitive impairment, while some males with FXS function nearly normally, and others are profoundly disabled (25). Several studies have reported "high-functioning" males with intellectual ability in the normal to borderline range (26, 27). Besides, one-third to half of females with a full *FMR1* mutation have intellectual functioning in the normal range, due to the masking effect of the normal X-chromosome *FMR1* allele (28). Numerous additional associated conditions and symptoms of variable severity can occur such as sleep disturbance, seizures, frequent otitis media, strabismus, and joint hyperlaxity (29). Interestingly, a FXS subgroup has been reported, characterized by hyperphagia, lack of satiation after meals and extreme obesity with a full, round face, small, broad hands/feet, and regional skin hyperpigmentation, referenced in literature as "Prader-Willi-like" (30, 31). This particular subgroup might point to the existence of several subgroups of patients with FXS which can be grouped based on phenotypic features and treated by targeting the corresponding underlying molecular differences. Prevalence of several conditions or symptoms associated to FXS are shown in **Tables 1, 2**.

This important degree of phenotypic heterogeneity in FXS probably mirrors a heterogeneous genetic background and the cellular-level involvement of various signaling pathways co-regulated by FMRP, such as PI3K and mTOR pathways (44). Moreover, genetic background plays an important role as shown in animal models (which would result in different patterns of expression of other proteins, including FMRP-interacting proteins) (45, 46), as well as (a) genetic consequences of variation on FMRP function at different levels including FMRP expression, and (b) FMRP effect on other genes mRNA transcripts by regulation of splicing, translation (through ribosome stalling), and RNA stability through the recognition of mRNA codon bias and N6-methyladenosine (m6A) modifications (15). In addition, individuals with FXS might show mosaicism at two different levels: (1) CGG repeat lengths, with some cells harboring fully expanded mutation alleles and other cells harboring more benign alleles; and (2) methylation levels, with some cells containing methylated *FMR1* alleles and other cells containing unmethylated

TABLE 1 | Reported prevalence of heterogeneous phenotypic features in FXS patients in several studies.

Global phenotypic features

Feature	References	Prevalence in FXS females	Prevalence in FXS males	Prevalence in FXS	Prevalence in FXS-negative controls
Adverse response to touch on the skin	Lachiewicz et al. (32)	–	–	61.1% (22/36)	18.9% (7/37)*
Aortic root dilatation	Ciaccio et al. (33)	–	–	25%	–
Brisk deep tendon reflexes	Lachiewicz et al. (32)	–	–	72.7% (26/36)	37.8% (14/37)*
Broad forehead	Lachiewicz et al. (32)	–	–	72.2% (26/36)	67.6% (25/37)–
Curvature of the spine	Lachiewicz et al. (32)	–	–	5.6% (2/36)	2.7% (1/37)–
Curved 5th finger	Lachiewicz et al. (32)	–	–	63.9% (23/36)	48.6% (18/37)–
Difficulty touching tongue to lips	Lachiewicz et al. (32)	–	–	75.9% (22/29)	27.6% (8/29)*
Difficulty pronouncing "puh–tuh–kuh"	Lachiewicz et al. (32)	–	–	72.4% (21/29)	46.2% (12/26)–
Difficulty moving the extended tongue from side to side	Lachiewicz et al. (32)	–	–	53.6% (15/28)	17.2% (5/29)*
Difficulty pronouncing "linoleum"	Lachiewicz et al. (32)	–	–	86.2% (25/29)	73.1% (19/26)–
EEG anomalies	Ciaccio et al. (33)	–	–	74%	–
Elongated/narrow face	Lachiewicz et al. (32)	–	–	83.3% (30/36)	45.9% (17/37)*
	Lubala et al. (34)			72.18% (109/151)	19.53% (533/2,728)*
	Ciaccio et al. (33)	–	–	83%	–
Epilepsy / Seizures	Lachiewicz et al. (32)	–	–	8.6% (3/35)	13.5% (5/37)–
	Berry–Kravis et al. (35)	4.8% (1/23)	13.3% (15/113)	11.7% (16/136)	–
	Berry–Kravis et al. (36)	6% (19/304)	14% (154/1,090)	12,41% (173/1,394)	–
	Kidd et al. (37)	3.2% ($n = 62$)	12.1% ($n = 198$)	10% ($n = 260$)	–
	Bailey et al. (38)	2.7% (7/259)	1.84% (18/976)	2.02% (25/1,235)	–
	Ciaccio et al. (33)	–	–	10 – 20%	–
	Symons et al. (39)	94% ($n = 51$)	81.8% ($n = 436$)	–	–
Flat feet	Lachiewicz et al. (32)	–	–	69.4% (25/36)	62.2% (23/37)–
	Yuskaitis et al. (40)	–	–	50% (75/150)	–
	Lubala et al. (34)	–	–	70.27% (26/37)	37.39% (43/115)*
	Ciaccio et al. (33)	–	–	29 – 69%	–
Gastrointestinal problems	Kidd et al. (37)	7% ($n = 62$)	12% ($n = 198$)	10.8% ($n = 260$)	–
	Ciaccio et al. (33)	–	–	31%	–
Hallucal crease	Lachiewicz et al. (32)	–	–	82.9% (29/35)	29.7% (11/37)*
Hyperextensible joints	Lachiewicz et al. (32)	–	–	100% (36/36)	75.7% (28/37)*
	Lubala et al. (34)	–	–	68.18% (150/220)	25.44% (849/3,336)*
Hand calluses	Lachiewicz et al. (32)	–	–	27.8% (10/36)	2.7% (2/37)*
Highly arched palate	Lachiewicz et al. (32)	–	–	94.4% (34/36)	70.3% (26/37)–
Horizontal palmar creases or distal axial triradii	Lachiewicz et al. (32)	–	–	25% (9/36)	13.5% (5/37)–
History of eye problems	Lachiewicz et al. (32)	–	–	45.7% (16/35)	21.6% (8/37)–
History of cleft lip/palate	Lachiewicz et al. (32)	–	–	2.8% (1/36)	0% (0/37)–
Hypotonia	Lachiewicz et al. (32)	–	–	72.2% (26/36)	48.6% (18/37)–
History of allergies	Lachiewicz et al. (32)	–	–	37.1% (13/35)	32.4% (12/37)–
History of spine curvature	Lachiewicz et al. (32)	–	–	2.8% (1/36)	2.7% (1/37)–
History of hernias	Lachiewicz et al. (32)	–	–	8.3% (3/36)	5.4% (2/37)–
History of > five ear infections/recurrent otitis media	Lachiewicz et al. (32)	–	–	97.2% (35/36)	91.9% (34/37)–
	Kidd et al. (37)	45.8% ($n = 62$)	54.7% ($n = 198$)	52.6% ($n = 260$)	–
	Ciaccio et al. (33)	–	–	47 – 97%	–
Inability to close eyes on request	Lachiewicz et al. (32)	–	–	14.5% (4/27)	0% (0/29)–
Joint hypermobility/Excessive laxity of the joints	Ciaccio et al. (33)	–	–	50%	–
	Yuskaitis et al. (40)	–	–	57% (85/150)	–
Large and prominent ears	Lubala et al. (34)	–	–	83.9% (173/206)	21.86% (756/3,458)*

(Continued)

TABLE 1 | Continued

Global phenotypic features					
Feature	**References**	**Prevalence in FXS females**	**Prevalence in FXS males**	**Prevalence in FXS**	**Prevalence in FXS-negative controls**
	Ciaccio et al. (33)	–	–	72 – 78%	–
	Lachiewicz et al. (32)	–	–	72.2% (26/36)	35.1% (13/27)*
Large testicles/Macroorchidism	Lachiewicz et al. (32)	–	–	62.9% (22/35)	29.7% (11/37)*
	Lubala et al. (34)	–	–	69.61% (129/181)	9.98% (291/2,915)*
	Ciaccio et al. (33)	–	–	63 – 95%	–
	Ciaccio et al. (33)	–	–	94%	–
Low birth weight	Kidd et al. (37)	12.7% ($n = 62$)	7.2% ($n = 198$)	8.6% ($n = 260$)	–
Macrocephaly/Head circumference > 50th centile	Lachiewicz et al. (32)	–	–	80.6% (29/36)	62.2% (23/37)–
	Ciaccio et al. (33)	–	–	81%	–
Mitral click	Lachiewicz et al. (32)	–	–	2.8% (1/36)	0% (0/37)–
Mitral valve anomalies	Ciaccio et al. (33)	–	–	3 – 12%	–
Mitral Valve prolapse	Kidd et al. (37)	1.7% ($n = 62$)	0.5% ($n = 198$)	0.8% ($n = 260$)	–
Motor tics	Kidd et al. (37)	6.7% ($n = 62$)	5.4% ($n = 198$)	5.7% ($n = 260$)	–
Nystagmus	Ciaccio et al. (33)	–	–	5 – 13%	–
Obesity	Ciaccio et al. (33)	–	–	53 – 61%	–
Obstructive sleep apnea	Kidd et al. (37)	7.1% ($n = 62$)	7.2% ($n = 198$)	7.2% ($n = 260$)	–
Ocular abnormalities	Lachiewicz et al. (32)	–	–	27.8% (10/36)	21.6% (8/37)–
Pale blue eyes	Lubala et al. (34)	–	–	57.14% (28/49)	7.25% (23/317)*
Prominent helices	Lachiewicz et al. (32)	–	–	66.7% (24/36)	40.5% (15/37)–
Prominent jaw	Ciaccio et al. (33)	–	–	80%	–
Pectus excavatum	Lachiewicz et al. (32)	–	–	50% (18/36)	29.7% (11/37)–
Pectus excavatum	Ciaccio et al. (33)	–	–	50%	–
Plantar crease	Lubala et al. (34)	–	–	85.71% (84/98)	22.91% (162/707)*
Refractive errors	Ciaccio et al. (33)	–	–	17 – 59%	–
Simply formed helices	Lachiewicz et al. (32)	–	–	27.8% (10/36)	13.5% (5/37)–
Strabismus	Kidd et al. (37)	12.9% ($n = 62$)	17.5% ($n = 198$)	16.4% ($n = 260$)	–
	Ciaccio et al. (33)	–	–	8 – 40%	–
Sleep problems	Kidd et al. (37)	29.8% ($n = 62$)	26% ($n = 198$)	26.9% ($n = 260$)	–
Spine deformity	Ciaccio et al. (33)	–	–	6 – 9%	–
Scoliosis	Yuskaitis et al. (40)	–	–	6.6% (10/150)	–
Skin soft and velvety	Lubala et al. (34)	–	–	88.37% (38/43)	5.24% (95/1,811)*
Soft skin over dorsum of hand	Lachiewicz et al. (32)	–	–	100% (35/35)	73% (27/37)*
Transverse palmar crease/Sydney lines	Lubala et al. (34)	–	–	26% (30/115)	9.77% (104/1,064)*

**Studies in which a significative difference was found between FXS patients and FXS-negative controls.*

FMR1 alleles. It is estimated that nearly half of individuals carrying the full *FMR1* mutation exhibit some sort of size and/or methylation mosaicism (47) (**Table 3**). Novel methods have improved detection of these alleles, which previously could only be detected by Southern blot (50). Size mosaicism, which is thought to arise due to CGG repeat instability, is normally observed as a combination of full mutation (>200 repeats) alleles with premutation alleles (>55) or rarely, even normal alleles. Methylation mosaicism can be observed in the form of unmethylated alleles, either showing a full mutation or a premutation allele. Both types of mosaicism will support the production of some FMRP, so individuals with size and /or methylation mosaicism might have less severe cognitive and behavioral defects than a patient with a full mutation and a completely methylated *FMR1* promoter, and in whom FMRP is markedly reduced or absent (19). Several authors have reported that male patients having full mutation with complete methylation had the lowest IQ scores and greatest physical involvement, in comparison to mosaic cases, although other studies have not observed this correlation (48, 51, 52). Correlation of degree of size and/or methylation mosaicism with other phenotypic features (seizures, hyperactivity, and autism) has been more difficult to establish. In a recent paper evaluating a cohort of male and female patients with FXS, male children carrying full *FMR1* mutation and expressing some degree of *FMR1* mRNA due to incomplete methylation had significantly

TABLE 2 | Reported prevalence of heterogeneous neurobehavioral features in FXS patients in several studies.

Neurobehavioral features					
Feature	**Study**	**Prevalence in FXS females**	**Prevalence in FXS males**	**Prevalence in FXS**	**Prevalence in FXS-negative controls**
Autism/Autistic-like behavior	Symons et al. (41)	6.17% (16/259)	4.71% (46/976)	5.02% (62/1,235)	–
	Symons et al. (39)	34% ($n = 51$)	55.4% ($n = 436$)	–	–
	Lubala et al. (34)	–	–	76.05% (162/213)	24.7% (854/3,457)*
	Budimirovic et al. (42)	1/5 (20%)	12/26 (46%)	–	–
	Kaufmann et al. (21)	18% ($n = 237$)	51% ($n = 237$)	42% ($n = 237$)	–
	Lewis et al. (43)	–	–	10/44 (22%)	–
Attention problems	Ciaccio et al. (33)	–	–	74 – 84%	–
	Symons et al. (41)	25.86% (67/259)	8.6% (84/976)	12.22% (151/1,235)	–
	Lubala et al. (34)	–	–	79.13% (91/115)	48.07% (511/1,063)*
	Symons et al. (39)	81.6% ($n = 51$)	87.4% ($n = 436$)	–	–
ADHD	Lubala et al. (34)	–	–	75.3% (122/162)	55.20% (870/1,576)*
Anxiety Disorder	Ciaccio et al. (33)	–	–	58 – 86%	–
	Budimirovic et al. (42)	–	–	100% (31/31)	–
Depression	Ciaccio et al. (33)	–	–	8 – 12%	–
	Symons et al. (41)	8.49% (22/259)	1.22% (12/976)	2.75% (34/1,235)	–
	Symons et al. (39)	29.2% ($n = 51$)	13.5% ($n = 436$)		
Developmental delay	Symons et al. (41)	24.71% (64/259)	9.83% (96/976)	12.95% (160/1,235)	–
Family history of intellectual disability	Lachiewicz et al. (32)	–	–	69.4% (25/36)	32.4% (12/37)*
	Lubala et al. (34)	–	–	80.97% (166/205)	23.61% (807/3,418)*
Gaze avoidance/ poor eye contact	Lachiewicz et al. (32)	–	–	83.3% (30/36)	51.4% (19/37)*
	Lubala et al. (34)			86.33% (139/161)	34.32% (517/1,506)*
Hand flapping	Lubala et al. (34)	–	–	58,59% (75/128)	29% (404/1,391)*
Hand–biting	Lubala et al. (34)	–	–	39.13% (45/115)	20.52% (218/1,062)*
Hyperactivity	Symons et al. (41)	11.58% (30/259)	6.76% (66/976)	7.77% (96/1,235)	–
	Ciaccio et al. (33)	–	–	50 – 66%	–
	Lubala et al. (34)	–	–	74.07% (120/162)	52.6% (829/1,576)*
	Symons et al. (39)	38.8% ($n = 51$)	71.9% ($n = 436$)		
	Symons et al. (41)	21.62% (56/259)	7.17% (70/976)	10.2% (126/1,235)	–
Perseverative speech	Lubala et al. (34)	–	–	66.45% (107/161)	46.04% (675/1,466)*
Previous diagnosis of intellectual disability	Lachiewicz et al. (32)	–	–	91.4% (32/35)	64.9% (24/37)*
Self-injurious behavior	Symons et al. (41)	3.86% (10/259)	4.2% (41/976)	4.12% (51/1,235)	–
Sleep problems	Ciaccio et al. (33)	–	–	30%	–
Tactilely defensive	Lubala et al. (34)	–	–	65% (108/166)	19.12% (626/3,274)*

**Studies in which a significative difference was found between FXS patients and FXS-negative controls.*

higher Autism Diagnostic Observational Schedule (ADOS) severity scores, compared to individuals with FXS carrying full *FMR1* mutation but with completely silenced *FMR1*. However, this association should be replicated in additional cohorts (53). In female premutation carriers, a study found that *FMR1* mRNA levels increase as the number of CGG repeats increases, suggesting that due to skewed X inactivation, mRNA levels tend to normalize in females when the number of CGG repeats increases, making clinical-genetic correlations more difficult to establish (54). While larger studies are needed, the expression levels of FMRP and methylation status of the *FMR1* gene have been correlated with cognitive ability (positive correlation for FMRP levels, negative correlation for methylation) (55), whereas little correlation between CGG repeat number and cognition is thought to exist. Given that residual levels of FMRP expression explain in part the heterogeneity in the FXS phenotype, the integration of diagnostic genomic data with *FMR1* mRNA measuring assays and more accurate FMRP profiles could clarify the relationships between genotypes, mRNA/protein expression and patient phenotypes. Deciphering these links would both improve disease prognostics and be useful to stratify patients with FXS for clinical trials (56).

In a very recent study, which assessed quantification of methylation, DNA, RNA, and FMRP protein levels in buccal and blood samples, methylation mosaicism was estimated to be slightly over 50%. Molecular-neurobehavioral correlations confirmed the inverse relationship between overall severity of the FXS phenotype and decrease in FMRP levels. Co-occurrence

TABLE 3 | Mosaicism features in FXS patient cohorts.

Study	Features
Nolin et al. (45)	**148 patients (males)** Full mutation/full methylation → 87/148 (59%) Mosaic pattern (full mutation/premutation) → 61/148 (41%)
Merenstein et al. (48)	**218 patients (males)** Full mutation/full methylation → 160/218 ∼73% Full mutation partial methylation → 12/218 ∼6% males Mosaic pattern (full mutation/premutation) v 46/218 ∼ 21% males
Rousseau et al. (49)	**1,051 patients (485 males, 283 females)** Full mutation/full methylation → 425 males (87.6%), 268 females (94.6%) Mosaic pattern (full mutation/premutation) → 60 males (12.3%), 15 females (5.3%)

of FXS with an autism diagnosis correlated significantly with 2-fold lower levels of FMRP specially in younger age- and IQ-adjusted males, compared to FXS without ASD, and patients with severe Intellectual Disability had even lower FMRP levels (42). In the same study, while Budimirovic et al. (42) also showed a high level of agreement in regard to FMRP protein levels between blood and buccal samples, but these findings have not always been replicated, and discordant presence of mutations between different tissues such as blood and skin have been reported (57–59). In parallel, it has been shown that repeat number can vary in different tissue types from the same individual carrying premutation alleles, suggesting that numbers obtained from blood mononuclear cells may not always directly translate to the brain. These results can complicate efforts to use blood results for clinical trial inclusion/exclusion criteria (60), as they suggest that FMRP levels in patients' cortical brain tissue might differ from levels observed in blood, at least for some FXS patients. Evidences of correlation between blood and brain FMRP levels in the individual subjects are limited; in particular, a study comparing brain and blood suggested that males with full mutations also have a certain level of mosaicism in brain tissue (61, 62). Further studies using blood and post-mortem brain samples will be required to shed light on this matter.

DIVERSITY OF RESPONSES TO THERAPEUTIC TREATMENTS

Despite two decades of preclinical work and several clinical trials targeting at least 10 different mechanisms associated with FXS pathogenesis (19), candidate drugs have been unsuccessful in reaching primary endpoints, and there are still no approved treatments to address the core symptoms of FXS. Well-studied pathophysiological mechanisms of FXS include excessive glutamatergic signaling, endocannabinoid system impaired signaling, voltage-gated ion channel dysfunction, GABAergic system inhibition, or excess of protein translation activity. While preclinical studies of drugs targeting these mechanisms in FXS mouse models (such as the widely used FMR1-KO model) provided positive results, translation to therapeutic use in human patients have not achieved the expected efficacy outcomes. Nevertheless, several clinical trials are ongoing, and several past clinical trials conducted in FXS did show clinical benefit in subsets of patients, providing hope for future drug development studies (63).

FMRP plays a critical role in mGluR (metabotropic glutamate receptor)-dependent long-term depression and its absence causes dysregulated synaptic function due to excessive and persistent protein synthesis in postsynaptic dendrites (64). Accordingly, in two clinical trials with mGlu5 receptor antagonists (fenobam, mavoglurant), which target an excessive activity of mGluR signaling downstream due to loss of inhibitory control by FMRP, improvement was observed in six and seven subjects out of 12 and 30 patients, respectively. In the fenobam study, a calmed behavior with improvement in eye contact, ability to interact, anxiety and/or motor overactivity was observed in nine out of 12 patients, pointing to an even higher rate, although further studies were not performed afterwards (65). In the initial mavoglurant clinical trial, seven out of 30 responder patients identified in a *post-hoc* analysis, had improved Aberrant Behavior Checklist—Community Edition scores (ABC-C) associated to complete *FMR1* promoter methylation and no detectable *FMR1* messenger RNA, while no improved response was shown in 18 patients with partial promoter methylation (66). These encouraging results prompted the design of two large IIb clinical trials (double—blind, placebo—controlled, performed in parallel, $n = 175$, 139), in which patients were also stratified by methylation status. Nevertheless, these studies did not report data on efficacy scores, nor evaluated the presence of positive responder patients (67). After negative results in the Phase III study, Novartis announced the discontinuation of the mavoglurant FXS development program (68). However, despite lack of success in past clinical trials, further studies continue to support a possible role of *FMR1* promoter methylation to stratify patients (69, 70).

On the other hand, in 2012 a first phase II clinical trial with arbaclofen (a $GABA_B$ receptor agonist) was performed based on successful preclinical model studies (71). Although no differences from placebo on the primary endpoint (irritability scores) were found, secondary outcome measures were associated with significant improvement. Using a novel ABC—Social Avoidance (ABC-SA) scale validated for the assessment of FXS, arbaclofen treatment was also associated with an overall beneficial effect. Furthermore, a *post-hoc* analysis focused on a subgroup of 27 subjects with more severe social impairment showed improvements in several scales compared to placebo treatment. The results were also more robust among subjects who met Diagnostic and Statistical Manual of Mental Disorders-Fourth Edition (DSM-IV) and Autism Diagnostic Interview-Revised (ADI-R) criteria for autistic disorder. Significantly more subjects were responders on the CGI-I scale when receiving arbaclofen vs. placebo (35 vs. 18% overall; 50 vs. 6% autism) in the autism subgroup, although again the ABC-C Irritability was not sensitive to these effects. Arbaclofen was then tested in two parallel phase 3 studies (randomized, double—blind, placebo—controlled, $n = 125$ and 172) in adults and children (35). These

two studies did not show benefit for arbaclofen over placebo for any measure (including the primary objective, showing efficacy reducing the ABC-CFX Social Avoidance score). Nevertheless, the child study showed that the highest dose group was associated to a benefit over placebo on ABC-CFX Irritability subscale and Parenting Stress Index, and results showed a trend toward improvement in social avoidance and hyperactivity subscales and CGI-I. Although additional studies with a larger cohort on higher doses would be required to confirm this finding, these results suggested potential dose- and age-related effects as well as a possible optimization of primary study endpoints, pointing toward a potential benefit of arbaclofen in future optimized clinical trials. Double-blind, randomized, placebo-controlled studies in FXS are also highly recommended to properly assess the sensitivity and specificity of clinical endpoints (63, 72, 73).

Clinical trials have also been performed for other drugs with limited success. Although not reaching primary outcomes, secondary endpoints or *post-hoc* analyses pointed to a considerable positive responder fraction of patients in several of them (**Table 4**), which suggest a potential benefit of these drugs that should be targeted and improved in future clinical trials. Only very recently, a promising phase-2 crossover study using phosphodiesterase-4D inhibitors (to increase cAMP production levels, which are reduced in patients with FXS) on a cohort of 30 FXS male adult patients showed improvement on cognition and daily function, while also meeting the primary objectives of safety and tolerability (78). Although these results shall be validated in future larger clinical trials, this study accounts for the importance of addressing cohort variability through the selection of meaningful endpoints capturing inter-individual variability.

DISCUSSION: THE NEED AND POTENTIAL IMPLICATIONS OF PRECISION MEDICINE IN FXS AND RELATED SYNDROMES

Although past efforts in clinical trials with FXS have mainly resulted in lack of success at meeting primary endpoints, these works have not been unfruitful. These large studies have built a base for future studies in FXS and other NDDs, in which several improvements should be incorporated, such as: use of optimized primary outcome measures (both neurobehavioral and related to cognition/language), discovery of novel prognosis and progression markers, administration of drugs from an earlier age and possibly longer times, performance of well-powered studies (as clinical trials with a lower number of participants were invariably positive in *post-hoc* analyses), and a better analysis and translation of preclinical mouse model studies to clinical studies in humans (63). A particular point of relevance for the future design of successful clinical trials in FXS is the characterization and further identification of subgroups of patients that respond to a specific drug treatment. While some studies have focused on establishing lists of minimal features to be screened to diagnose patients with FXS (32, 34), less focus has been put on the identification of subgroups of patients with FXS according to their phenotypic or molecular characteristics. Improved patient stratification would most likely help to pair pharmacologic agents with patients most likely to respond positively to such therapeutic treatments.

Besides *FMR1* methylation levels and selection on isolated clinical features (such as high-functioning individuals or Prader-Willi-like subphenotype mentioned above), other stratification strategies based on precision medicine have been suggested and might be implemented in the future. A recent work reported the use of structural brain growth as a marker to identify clinically significative subgroups. Using topological data analysis on T1-weighted anatomical MRI data from 42 FXS children patients, researchers identified two previously unknown large subgroups of patients. *Post-hoc* analyses between these groups demonstrated that one group was consistently higher functioning on cognition, adaptive functioning, and autism severity scores. As pointed by the authors, anatomical MRI data analysis might become a useful method to define subtypes within other neuropsychiatric disorders (79). In another recent study, electronic health records from more than one million people were mined to investigate health characteristics of individuals clinically diagnosed with FXS. This resulted into (1) the identification of previously unnoticed significative co-occurring health conditions in patients with FXS (heart and circulatory disorders, medication side effects, and among others), and (2) the development of a predictive model to identify patients with FXS in the general population without using any genetic data, successfully identifying cases 5 years prior to clinical diagnosis of FXS (80). While this AI-assisted diagnosis method was instrumental to identify cases in the general population prior to the onset of more severe symptoms (80), no computational methods have been oriented toward stratifying patients with FXS into more homogenous subgroups, which is the first step needed to enrich future clinical trials in FXS with responder patients. Recently, the use of patient-derived induced pluripotent stem cells was proposed to model the disease in a patient-specific manner and to develop new therapeutic opportunities (81); nevertheless, to our knowledge, there is still no clinical trials in FXS involving therapeutic treatments developed using patient-derived induced pluripotent stem cells.

Accurate stratification of patients is expected to be crucial for the development of efficient drug treatments in FXS. Early applications of systems biology driven *in silico* drug repositioning in FXS were conducted without specific focus on genetic heterogeneity (HealX drove the advancement of a FXS repurposing discovery effort in collaboration with FRAXA) (82). In order to overcome this limitation, systems biology and precision-medicine based computational aided modeling are emerging in the NDD space both in academic and industry setting. These approaches offer new potential for novel subgroup characterization and further identification of FXS and other NDD patients with stronger biological potential to respond to specific drug candidates. For instance, STALICLA's DEPI platform was recently used to identify subsets of clinical features that significantly correlate with the molecular responses induced by arbaclofen in cellular models, which could support the identification of patients predicted to improve under arbaclofen's treatment. An observational study involving patients with FXS which had participated in previous clinical trials with arbaclofen

TABLE 4 | FXS clinical trials showing positive responder results.

References	Drug	Phase	Positive responders %	Primary endpoint	Scales used to define or measure improvement in responders	Negative responders
Berry-Kravis et al. (65)	Fenobam (mGluR5 antagonist)	IIa, open-label study	50% (6/12: 4/6 males and 2/6 females)	-	PPI (improved over test–retest controls)	No adverse effects reported
Jacquemont et al. (66)	Mavoglurant (mGluR5 antagonist)	IIa	23.33% (7/30 males)	ABC-C (not attained)	ABC-C of selected patients after *post-hoc* analysis	No neurobehavioral adverse effects reported (24/ 30 mild to moderate fatigue and headache)
Berry-Kravis et al. (71)	Arbaclofen ($GABA_B$ receptor agonist)	II	47.6% (10/21 patients with increased social impairment, most of them males) vs. 8.7% (placebo)	ABC-I (not attained)	Defined by CGI-I, ABC_{-LSW}	No neuro behavioral adverse effects reported (8% cases of sedation and of headache)
Berry-Kravis et al. (35)	Arbaclofen ($GABA_B$ receptor agonist)	III	35% in children patients, vs. 21% (placebo)	ABC_{-CFX} (not attained)	Defined by CGI-I, ABC_{-CFX}	Irritability, agitation, anxiety, hyperactivity (45 vs. 40% controls in adults, ∼36 vs. 34% in children), other extra neurological features
Erickson et al. (74)	Acamprosate	III open-label study	75% (9/12 subjects)	CGI-I	CGI-I	irritability, repetitive behavior
Berry-Kravis et al. (75)	Lithium	IIa	86% (13/15, ABC-C), 86% (13/15, CGI), 80% (12/15, VAS),	ABC-C Irritability (not attained)	ABC-C, CGI, VAS	Irritability, appetite changes, bed wetting, constipation or diarrhea, headache, polydipsia, polyuria, sleep problems, tiredness, vomiting, high TSH
Paribello et al. (76)	Minocycline	IIa (open-label)	63% (12/19)	ABC-C	ABC-C, CGI, VAS	Minor diarrhea, seroconversion to a positive ANA
Greiss Hess et al. (77)	Sertraline		52% (13/25 sertraline) vs. 44% (12/27 control) improved symptoms	CGI-I, MSEL-EL	MSEL-EL, *post-hoc* analysis	Upper respiratory infection, diarrhea, and gastrointestinal issues
Berry-Kravis et al. (78)	BPN14770	II, two-way crossover study	NA	Safety, tolerability	National Institutes of Health-Toolbox Cognition Battery (NIH-TCB) and Test of Attentional Performance for Children (KiTAP)	There was no TEAE (treatment-emergent adverse events) judged by the investigator to be at least possibly related to treatment with BPN14770.

ABC-C, Aberrant Behavior Checklist–Community Edition; ABC-I, ABC–Irritability; ABC-LSW, ABC—Lethargy/Social Withdrawal; ABC-CFX, ABC- Fragile X specific; MSEL-EL, Mullen Scales of Early Learning, Expressive Language; ET, eye tracking; CGI-I, Clinical Global Impression-Improvement; PPI, Prepulse Inhibition; VAS, Visual Analog Scale.

is currently ongoing, with the main goal to provide clinical validation for this defined subgroup of patients with FXS. Importantly, other NDD patients (without a FXS diagnose) might qualify to fit into this target population and potentially benefit from the same compound.

In summary, well-defined NDD syndromes such as FXS (the most frequent monogenic cause of intellectual disability and ASD among NDDs) constitute a first step to switch from a behavioral to a molecular-centered based diagnosis. Precision medicine in FXS will continue to be necessary to (1) define more precisely subgroups of patients for clinical trials, and (2) define drug responder profiles that can associate patients with different genetic backgrounds to a same candidate drug. Importantly, these targeted populations of patients might expand beyond the FXS indication.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

REFERENCES

1. American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders*. American Psychiatric Association, Michigan, Ann Arbor, MI, USA. (2013). doi: 10.1176/appi.books.9780890425596
2. Moreno-De-Luca A, Myers SM, Challman TD, Moreno-De-Luca D, Evans DW, Ledbetter DH. Developmental brain dysfunction: revival and expansion of old concepts based on new genetic evidence. *Lancet Neurol.* (2013) 12:406–14. doi: 10.1016/S1474-4422(13)70011-5
3. Hansen BH, Oerbeck B, Skirbekk B, Petrovski BÉ, Kristensen H. Neurodevelopmental disorders: prevalence and comorbidity in children referred to mental health services. *Nord J Psychiatry.* (2018) 72:285–91. doi: 10.1080/08039488.2018.1444087
4. Gillberg C. The ESSENCE in child psychiatry: early symptomatic syndromes eliciting neurodevelopmental clinical examinations. *Res Dev Disabil.* (2010) 31:1543–51. doi: 10.1016/j.ridd.2010.06.002
5. Wasilewska J, Klukowski M. Gastrointestinal symptoms and autism spectrum disorder: links and risks – a possible new overlap syndrome. *Pediatr Heal Med Ther.* (2015) 6:153. doi: 10.2147/PHMT.S85717
6. Kedem S, Yust-Katz S, Carter D, Levi Z, Kedem R, Dickstein A, et al. Attention deficit hyperactivity disorder and gastrointestinal morbidity in a large cohort of young adults. *World J Gastroenterol.* (2020) 26:6626. doi: 10.3748/wjg.v26.i42.6626
7. Kang V, Wagner GC, Ming X. Gastrointestinal dysfunction in children with autism spectrum disorders. *Autism Res.* (2014) 7:501–6. doi: 10.1002/aur.1386
8. Magalhães ES, Pinto-Mariz F, Bastos-Pinto S, Pontes AT, Prado EA, deAzevedo LC. Immune allergic response in Asperger syndrome. *J Neuroimmunol.* (2009) 216:108–12. doi: 10.1016/j.jneuroim.2009.09.015
9. Meltzer A, Van de Water J. The role of the immune system in autism spectrum disorder. *Neuropsychopharmacol.* (2016) 42:284–98. doi: 10.1038/npp.2016.158
10. Kochinke K, Zweier C, Nijhof B, Fenckova M, Cizek P, Honti F, et al. Systematic phenomics analysis deconvolutes genes mutated in intellectual disability into biologically coherent modules. *Am J Hum Genet.* (2016) 98:149–64. doi: 10.1016/j.ajhg.2015.11.024
11. Parenti I, Rabaneda LG, Schoen H, Novarino G. Neurodevelopmental disorders: from genetics to functional pathways. *Trends Neurosci.* (2020) 43:608–21. doi: 10.1016/j.tins.2020.05.004
12. Allanson JE, Roberts AE. *Noonan Syndrome*. Seattle, WA: University of Washington (1993). Available online at: http://www.ncbi.nlm.nih.gov/pubmed/20301303 (accessed April 19, 2021).
13. Christodoulou J, Ho G. *MECP2-Related Disorders*. Seattle, WA: University of Washington (1993). Available online at: http://www.ncbi.nlm.nih.gov/pubmed/20301670 (accessed April 19, 2021).
14. den Hoed J, de Boer E, Voisin N, Dingemans AJM, Guex N, Wiel L, et al. Mutation-specific pathophysiological mechanisms define different neurodevelopmental disorders associated with SATB1 dysfunction. *Am J Hum Genet.* (2021) 108:346–56. doi: 10.1016/j.ajhg.2021.01.007
15. Richter JD, Zhao X. The molecular biology of FMRP: new insights into fragile X syndrome. *Nat Rev Neurosci.* (2021) 22:209–22. doi: 10.1038/s41583-021-00432-0
16. Martin JP, Bell J. A pedigree of mental defect showing. Sex-linkage. *J Neurol Neurosurg Psychiatry.* (1943) 6:154–7. doi: 10.1136/jnnp.6.3-4.154
17. Lubs HA. A marker X chromosome. *Am J Hum Genet.* (1969) 21:231–44. Available online at: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1706424/ (accessed May 31, 2021).
18. Fu YH, Kuhl DPA, Pizzuti A, Pieretti M, Sutcliffe JS, Richards S, et al. Variation of the CGG repeat at the fragile X site results in genetic instability: resolution of the Sherman paradox. *Cell.* (1991) 67:1047–58. doi: 10.1016/0092-8674(91)90283-5
19. Hagerman RJ, Berry-Kravis E, Hazlett HC, Bailey DB, Moine H, Kooy RF, et al. Fragile X syndrome. *Nat Rev Dis Prim.* (2017) 3:17065. doi: 10.1038/nrdp.2017.65
20. Gross C, Hoffmann A, Bassell GJ, Berry-Kravis EM. Therapeutic strategies in fragile X syndrome: from bench to bedside and back. *Neurotherapeutics.* (2015) 12:584–608. doi: 10.1007/s13311-015-0355-9
21. Kaufmann WE, Kidd SA, Andrews HF, Budimirovic DB, Esler A, Haas-Givler B, et al. Autism spectrum disorder in fragile X syndrome: cooccurring conditions and current treatment. *Pediatrics.* (2017) 139:S194–206. doi: 10.1542/peds.2016-1159F
22. Niu M, Han Y, Dy ABC, Du J, Jin H, Qin J, et al. Hagerman RJ. Autism symptoms in fragile X syndrome. *J Child Neurol.* (2017) 32:903–9. doi: 10.1177/0883073817712875
23. Budimirovic DB, Kaufmann WE. What can we learn about autism from studying fragile X syndrome? *Dev Neurosci.* (2011) 33:379–94. doi: 10.1159/000330213
24. Budimirovic DB, Bukelis I, Cox C, Gray RM, Tierney E, Kaufmann WE. Autism spectrum disorder in Fragile X syndrome: differential contribution of adaptive socialization and social withdrawal. *Am J Med Genet A.* (2006) 140A:1814–1826. doi: 10.1002/ajmg.a.31405
25. Garber KB, Visootsak J, Warren ST. Fragile X syndrome. *Eur J Hum Genet.* (2008) 16:666–72. doi: 10.1038/ejhg.2008.61
26. Hagerman RJ, Hull CE, Safanda JF, Carpenter I, Staley LW, O'Connor RA, et al. High functioning fragile X males: Demonstration of an unmethylated fully expanded FMR-1 mutation associated with protein expression. *Am J Med Genet.* (1994) 51:298–308. doi: 10.1002/ajmg.1320510404
27. Basuta K, Schneider A, Gane L, Polussa J, Woodruff B, Pretto D, et al. High functioning male with fragile X syndrome and fragile X-associated tremor/ataxia syndrome. *Am J Med Genet A.* (2015) 167:2154–61. doi: 10.1002/ajmg.a.37125
28. Chaste P, Betancur C, Gérard-Blanluet M, Bargiacchi A, Kuzbari S, Drunat S, et al. High-functioning autism spectrum disorder and fragile X syndrome: Report of two affected sisters. *Mol Autism.* (2012) 3:5. doi: 10.1186/2040-2392-3-5
29. Sherman SL, Kidd SA, Riley C, Berry-Kravis E, Andrews HF, Miller RM, et al. Forward: A registry and longitudinal clinical database to study fragile X syndrome. *Pediatrics.* (2017) 139:S183–93. doi: 10.1542/peds.2016-1159E
30. de Vries BBA, Fryns JP, Butler MG, Canziani F, Wesby-van Swaay E, van Hemel JO, et al. Clinical and molecular studies in fragile X patients with a Prader-Willi-like phenotype. *J Med Genet.* (1993) 30:761–6. doi: 10.1136/jmg.30.9.761
31. Muzar Z, Lozano R, Kolevzon A, Hagerman RJ. The neurobiology of the prader-willi phenotype of fragile x syndrome. *Intractable Rare Dis Res.* (2016) 5:255–61. doi: 10.5582/irdr.2016.01082
32. Lachiewicz AM, Dawson DV, Spiridigliozzi GA. Physical characteristics of young boys with fragile X syndrome: Reasons for difficulties in making a diagnosis in young males. *Am J Med Genet.* (2000) 92:229–36. doi: 10.1002/(SICI)1096-8628(20000605)92:4<229::AID-AJMG1>3.0.CO;2-K
33. Ciaccio C, Fontana L, Milani D, Tabano S, Miozzo M, Esposito S. Fragile X syndrome: a review of clinical and molecular diagnoses. *Ital J Pediatr.* (2017) 43:39. doi: 10.1186/s13052-017-0355-y
34. Lubala TK, Lumaka A, Kanteng G, Mutesa L, Mukuku O, Wembonyama S, et al. Fragile X checklists: A meta-analysis and development of a simplified universal clinical checklist. *Mol Genet Genomic Med.* (2018) 6:526–32. doi: 10.1002/mgg3.398
35. Berry-Kravis E, Hagerman R, Visootsak J, Budimirovic D, Kaufmann WE, Cherubini M, et al. Arbaclofen in fragile X syndrome: results of phase 3 trials. *J Neurodev Disord.* (2017) 9:6. doi: 10.1186/s11689-016-9181-6
36. Berry-Kravis E, Raspa M, Loggin-Hester L, Bishop E, Holiday D, Bailey DB. Seizures in fragile X syndrome: characteristics and comorbid diagnoses. *Am J Intellect Dev Disabil.* (2010) 115:461–72. doi: 10.1352/1944-7558-115.6.461
37. Kidd SA, Lachiewicz A, Barbouth D, Blitz RK, Delahunty C, McBrien D, et al. Fragile X syndrome: a review of associated medical problems. *Pediatrics.* (2014) 134:995–1005. doi: 10.1542/peds.2013-4301
38. Bailey DB, Raspa M, Olmsted M, Holiday DB. Co-occurring conditions associated with FMR1 gene variations: findings from a national parent survey. *Am J Med Genet A.* (2008) 146A:2060–9. doi: 10.1002/ajmg.a.32439
39. Symons FJ, Byiers BJ, Raspa M, Bishop E, Bailey DB. Self-injurious behavior and fragile X syndrome: findings from the national fragile X survey. *Am J Intellect Dev Disabil.* (2010) 115:473–81. doi: 10.1352/1944-7558-115.6.473
40. Yuskaitis CJ, Mines MA, King MK, Sweatt JD, Miller CA, Jope RS. Lithium ameliorates altered glycogen synthase kinase-3 and behavior in a mouse model of Fragile X syndrome. *Biochem Pharmacol.* 79, 632–646. doi: 10.1016/j.bcp.2009.09.023

41. Symons FJ, Shinde SK, Gilles E. Perspectives on pain and intellectual disability. *J Intellect Disabil Res.* (2008) 52:275–86. doi: 10.1111/j.1365-2788.2007.01037.x
42. Budimirovic DB, Schlageter A, Filipovic-Sadic S, Protic DD, Bram E, Mahone EM, et al. A genotype-phenotype study of high-resolution FMR1 nucleic acid and protein analyses in fragile X patients with neurobehavioral assessments. *Brain Sci.* (2020) 10:1–25. doi: 10.3390/brainsci10100694
43. Lewis P, Abbeduto L, Murphy M, Richmond E, Giles N, Bruno L, et al. Psychological well-being of mothers of youth with fragile X syndrome: syndrome specificity and within-syndrome variability. *J Intellect Disabil Res.* (2006) 50(Pt 12):894–904. doi: 10.1111/j.1365-2788.2006.00907.x
44. Wang T, Bray SM, Warren ST. New perspectives on the biology of fragile X syndrome. *Curr Opin Genet Dev.* (2012) 22:256–63. doi: 10.1016/j.gde.2012.02.002
45. Kennedy T, Rinker D, Broadie K. Genetic background mutations drive neural circuit hyperconnectivity in a fragile X syndrome model. *BMC Biol.* (2020) 18:817. doi: 10.1186/s12915-020-00817-0
46. Spencer CM, Alekseyenko O, Hamilton SM, Thomas AM, Serysheva E, Yuva-Paylor LA, et al. Modifying behavioral phenotypes in Fmr1KO mice: genetic background differences reveal autistic-like responses. *Autism Res.* (2011) 4:40–56. doi: 10.1002/aur.168
47. Nolin SL, Glicksman A, Houck GE, Brown WT, Dobkin CS. Mosaicism in fragile X affected males. *Am J Med Genet.* (1994) 51:509–12. doi: 10.1002/ajmg.1320510444
48. Merenstein SA, Sobesky WE, Taylor AK, Riddle JE, Tran HX, Hagerman RJ. Molecular-clinical correlations in males with an expanded FMR1 mutation. *Am J Med Genet.* (1996) 64:388–94. doi: 10.1002/(SICI)1096-8628(19960809)64:2<388::AID-AJMG31>3.0.CO;2-9
49. Rousseau F, Heitz, D, Tarleton, MacPherson J, Malmgren J, Dahl N, et al. A multicenter study on genotype-phenotype correlations in the fragile X syndrome, using direct diagnosis with probe StB12.3: the first 2,253 cases. *Am J Hum Genet.* (1994) 55:225–37.
50. Aliaga SM, Slater HR, Francis D, Sart D, Du D, Li X, et al. Identification of males with cryptic fragile x alleles by methylation-Specific quantitative melt analysis. *Clin Chem.* (2016) 62:343–52. doi: 10.1373/clinchem.2015.244681
51. Pretto D, Yrigollen CM, Tang HT, Williamson J, Espinal G, Iwahashi CK, et al. Clinical and molecular implications of mosaicism in FMR1 full mutations. *Front Genet.* (2014) 5:1–11. doi: 10.3389/fgene.2014.00318
52. Jiraanont P, Kumar M, Tang HT, Espinal G, Hagerman PJ, Hagerman RJ, et al. Size and methylation mosaicism in males with Fragile X syndrome. *Expert Rev Mol Diagn.* (2017) 17:1023–32. doi: 10.1080/14737159.2017.1377612
53. Baker EK, Arpone M, Aliaga SM, Bretherton L, Kraan CM, Bui M, et al. Incomplete silencing of full mutation alleles in males with fragile X syndrome is associated with autistic features. *Mol Autism.* (2019) 10:1–13. doi: 10.1186/s13229-019-0271-7
54. García-Alegría E, Ibáñez B, Mínguez M, Poch M, Valiente A, Sanz-Parra A, et al. Analysis of FMR1 gene expression in female premutation carriers using robust segmented linear regression models. *RNA.* (2007) 13:756–62. doi: 10.1261/rna.206307
55. Loesch DZ, Huggins RM, Hagerman RJ. Phenotypic variation and FMRP levels in fragile X. *Ment Retard Dev Disabil Res Rev.* (2004) 10:31–41. doi: 10.1002/mrdd.20006
56. Budimirovic DB, Berry-Kravis E, Erickson CA, Hall SS, Hessl D, Reiss AL, et al. Updated report on tools to measure outcomes of clinical trials in fragile X syndrome. *J Neurodev Disord.* (2017) 9:14. doi: 10.1186/s11689-017-9193-x
57. Dobkin CS, Nolin SL, Cohen I, Sudhalter V, Bialer MG, Ding XH, et al. Tissue differences in fragile X mosaics: Mosaicism in blood cells may differ greatly from skin. *Am J Med Genet.* (1996) 64:296–301. doi: 10.1002/(SICI)1096-8628(19960809)64:2<296::AID-AJMG13>3.0.CO;2-A
58. Maddalena A, Yadvish KN, Spence WC, Howard-Peebles PN. A fragile X mosaic male with a cryptic full mutation detected in epithelium but not in blood. *Am J Med Genet.* (1996) 64:309–12. doi: 10.1002/(SICI)1096-8628(19960809)64:2<309::AID-AJMG15>3.0.CO;2-J
59. Tassone F, Hagerman RJ, Gane LW, Taylor AK. Strong similarities of theFMR1 mutation in multiple tissues: Postmortem studies of a male with a full mutation and a male carrier of a premutation. *Am J Med Genet.* (1999) 84:240–4. doi: 10.1002/(SICI)1096-8628(19990528)84:3<240::AID-AJMG15>3.0.CO;2-B
60. Schaefer TL, Davenport MH, Erickson CA. Emerging pharmacologic treatment options for fragile X syndrome. *Appl Clin Genet.* (2015) 8:75–93. doi: 10.2147/TACG.S35673
61. Pretto DI, Hunsaker MR, Cunningham CL, Greco CM, Hagerman RJ, Noctor SC, et al. Intranuclear inclusions in a fragile X mosaic male. *Transl Neurodegener.* (2013) 2:10. doi: 10.1186/2047-9158-2-10
62. De Graaff E, Willemsen R, Zhong N, De Die-Smulders CEM, Brown WT, Freling G, et al. Instability of the CGG repeat and expression of the FMRI protein in a male fragile X patient with a lung tumor. *Am J Hum Genet.* (1995) 57:609–18.
63. Berry-Kravis EM, Lindemann L, Jønch AE, Apostol G, Bear MF, Carpenter RL, et al. Drug development for neurodevelopmental disorders: lessons learned from fragile X syndrome. *Nat Rev Drug Discov.* (2018) 17:280–98. doi: 10.1038/nrd.2017.221
64. Santoro MR, Bray SM, Warren ST. Molecular mechanisms of fragile X syndrome: a twenty-year perspective. *Annu Rev Pathol Mech Dis.* (2012) 7:219–45. doi: 10.1146/annurev-pathol-011811-132457
65. Berry-Kravis E, Hessl D, Coffey S, Hervey C, Schneider A, Yuhas J, et al. A pilot open label, single dose trial of fenobam in adults with fragile X syndrome. *J Med Genet.* (2009) 46:266–71. doi: 10.1136/jmg.2008.063701
66. Jacquemont S, Curie A, Des Portes V, Torrioli MG, Berry-Kravis E, Hagerman RJ, et al. Epigenetic modification of the FMR1 gene in fragile X syndrome is associated with differential response to the mGluR5 antagonist AFQ056. *Sci Transl Med.* (2011) 3:1708. doi: 10.1126/scitranslmed.3001708
67. Berry-Kravis E, Des Portes V, Hagerman R, Jacquemont S, Charles P, Visootsak J, et al. Mavoglurant in fragile X syndrome: results of two randomized, double-blind, placebo-controlled trials. *Sci Transl Med.* (2016) 8:aab4109. doi: 10.1126/scitranslmed.aab4109
68. FRAXA Research Foundation. *Fragile X Syndrome Research & Treatment. FRAXA Research Foundation - Finding a Cure for Fragile X*. Available online at: https://www.fraxa.org/ (accessed April 23, 2021).
69. Baker EK, Arpone M, Kraan C, Bui M, Rogers C, Field M, et al. FMR1 mRnA from full mutation alleles is associated with ABc-c fX scores in males with fragile X syndrome. *Sci Rep.* (2020) 10:11701. doi: 10.1038/s41598-020-68465-6
70. Zynerba Pharmaceuticals. *Zynerba Pharmaceuticals Provides Regulatory Update on Zygel™ in Fragile X Syndrome - Zynerba*. Available online at: https://zynerba.com/zynerba-pharmaceuticals-provides-regulatory-update-on-zygel-in-fragile-x-syndrome/ (accessed April 23, 2021).
71. Berry-Kravis EM, Hessl D, Rathmell B, Zarevics P, Cherubini M, Walton-Bowen K, et al. Effects of STX209 (Arbaclofen) on neurobehavioral function in children and adults with fragile X syndrome: a randomized, controlled, phase 2 trial. *Sci Transl Med.* (2012) 4:152ra127. doi: 10.1126/scitranslmed.3004214
72. Luu S, Province H, Berry-Kravis E, Hagerman R, Hessl D, Vaidya D, et al. Response to placebo in fragile X syndrome clinical trials: an initial analysis. *Brain Sci.* (2020) 10:629. doi: 10.3390/brainsci10090629
73. Jacquemont S, Berry-Kravis E, Hagerman R, Von Raison F, Gasparini F, Apostol G, et al. The challenges of clinical trials in fragile X syndrome. *Psychopharmacology.* (2014) 231:1237–50. doi: 10.1007/s00213-013-3289-0
74. Erickson CA, Wink LK, Ray B, Early MC, Stiegelmeyer E, Mathieu-Frasier L, et al. Impact of acamprosate on behavior and brain-derived neurotrophic factor: an open-label study in youth with fragile X syndrome. *Psychopharmacology.* (2013) 228:75–84. doi: 10.1007/s00213-013-3022-z
75. Berry-Kravis E, Sumis A, Kim OK, Lara R, Wuu J. Characterization of potential outcome measures for future clinical trials in fragile X syndrome. *J Autism Dev Disord.* (2008) 38:1751–7. doi: 10.1007/s10803-008-0564-8
76. Paribello C, Tao L, Folino A, Berry-Kravis E, Tranfaglia M, Ethell IM, et al. Open-label add-on treatment trial of minocycline in fragile X syndrome. *MC Neurol.* (2010) 10:91. doi: 10.1186/1471-2377-10-91

77. Greiss Hess L, Fitzpatrick SE, Nguyen DV, Chen Y, Gaul KN, Schneider A, et al. A randomized, double-blind, placebo-controlled trial of low-dose sertraline in young children with fragile X syndrome. *J Dev Behav Pediatr.* (2016) 37:619–28. doi: 10.1097/DBP.0000000000000334
78. Berry-Kravis EM, Harnett MD, Reines SA, Reese MA, Ethridge LE, Outterson AH, et al. Inhibition of phosphodiesterase-4D in adults with fragile X syndrome: a randomized, placebo-controlled, phase 2 clinical trial. *Nat Med.* (2021) 27:862–70. doi: 10.1038/s41591-021-01321-w
79. Bruno JL, Romano D, Mazaika P, Lightbody AA, Hazlett HC, Piven J, et al. Longitudinal identification of clinically distinct neurophenotypes in young children with fragile X syndrome. *Proc Natl Acad Sci USA.* (2017) 114:10767–72. doi: 10.1073/pnas.1620994114
80. Movaghar A, Page D, Scholze D, Hong J, Dawalt LS, Kuusisto F, et al. Artificial intelligence-assisted phenotype discovery of fragile X syndrome in a population-based sample. *Genet Med.* 23:1273–80. doi: 10.1038/s41436-021-01144-7
81. Mor-Shaked H, Eiges R. Modeling fragile? syndrome using human pluripotent stem cells. *Genes.* (2016) 7:100077. doi: 10.3390/genes7100077
82. Tranfaglia MR, Thibodeaux C, Mason DJ, Brown D, Roberts I, Smith R, et al. Repurposing available drugs for neurodevelopmental disorders: the fragile X experience. *Neuropharmacology.* (2019) 147:74–86. doi: 10.1016/j.neuropharm.2018.05.004

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Trajectories of Heart Activity Across Infancy to Early Childhood Differentially Predict Autism and Anxiety Symptoms in Fragile X Syndrome

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Background: Fragile X syndrome (FXS) is a monogenic disorder characterized by high rates of autism spectrum disorder (ASD) and anxiety. A longstanding "hyperarousal hypothesis" in FXS has argued that ANS dysfunction underpins many symptoms of FXS. However, the developmental onset and trajectory of ANS dysfunction, as well as the consequences of ANS dysfunction on later psychiatric symptoms, remain poorly understood in FXS. Insight into the emergence, trajectory, and consequences of ANS dysfunction across early development in FXS has critical implications for prevention, intervention, and optimal outcomes in both typical and atypical development. This longitudinal study investigated whether and when males with FXS evidence atypical ANS function from infancy through early childhood, and how trajectories of ANS function across infancy and early childhood predict ASD and anxiety symptom severity later in development.

Methods: Participants included 73 males with FXS and 79 age-matched typically developing (TD) males. Baseline heart activity was recorded at multiple assessments between 3 and 83 months of age, resulting in 372 observations. General arousal and parasympathetic activity were indexed via interbeat interval (IBI) and respiratory sinus arrhythmia (RSA), respectively. ASD and anxiety symptoms were assessed at 36 months of age or later in a subgroup of participants (FXS $n = 28$; TD $n = 25$).

Results: Males with FXS exhibited atypical patterns of developmental change in ANS function across infancy and early childhood. As a result, ANS dysfunction became progressively more discrepant across time, with the FXS group exhibiting significantly shorter IBI and lower RSA by 29 and 24 months of age, respectively. Shorter IBI at 24 months and a flatter IBI slope across development predicted elevated anxiety symptoms, but not ASD symptoms, later in childhood in both FXS and TD males. Reduced RSA at 24 months predicted elevated ASD symptoms, but not anxiety symptoms, in both groups. Developmental change in RSA across early development did not predict later anxiety or ASD symptoms.

Conclusion: This is the first longitudinal study to examine the "hyperarousal hypothesis" in infants and young children with FXS. Findings suggest that hyperarousal (i.e., shorter IBI, lower RSA) is evident in males with FXS by 24–29 months of age. Interestingly, unique aspects of early ANS function differentially relate to later ASD and anxiety symptoms. General arousal, indexed by shorter IBI that becomes progressively more discrepant from TD controls, predicts later anxiety symptoms. In contrast, parasympathetic-related factors, indexed by lower levels of RSA, predict ASD symptoms. These findings support the "hyperarousal hypothesis" in FXS, in that ANS dysfunction evident early in development predicts later-emerging symptoms of ASD and anxiety. This study also have important implications for the development of targeted treatments and interventions that could potentially mitigate the long-term effects of hyperarousal in FXS.

Keywords: autonomic nervous system, respiratory sinus arrhythmia, interbeat interval, hyperarousal, fragile X syndrome, autism spectrum disorder, anxiety

INTRODUCTION

The autonomic nervous system (ANS) is primarily responsible for maintaining physiological balance in the human body by mobilizing the body's resources in the face of challenge or stress and facilitating physiological rest and relaxation when that challenge or stress has abated (1–4). When the sympathetic nervous system (SNS) and parasympathetic nervous system (PNS) of the ANS are balanced and functioning optimally, our bodies respond flexibly to and recover quickly from stress (5). Cardiac indices of ANS function provide unique, moment-to-moment insight into SNS and PNS influences on the heart (6, 7); specifically, interbeat interval (IBI), the time between successive heartbeats, indexes heart rate (HR) and is a reflection of general arousal, which is influenced by both the PNS and SNS (5, 6, 8). Alternatively, respiratory sinus arrhythmia (RSA), the variability in IBI that corresponds with spontaneous breathing, indexes heart rate variability (HRV) and more specifically PNS regulation of the heart via the vagal nerve (2, 4, 9–12). The ANS underlies processes related to attention as well as emotional and behavioral regulation (5, 13–15). Furthermore, at baseline and in the absence of challenge, stronger vagal regulation of the heart, reflected in higher RSA, is thought to support social engagement and has been associated with flexible response to stress, adaptive functioning, and prosocial behavior (4, 16). Thus, chronic imbalance between the two branches of the ANS has significant cascading effects on emotional, behavioral, and cognitive development as well as overall physical well-being (17, 18). Given the connection between the ANS and emotion regulation, it is unsurprising that ANS dysfunction has been linked to several psychiatric conditions characterized by emotion regulation difficulties, such as anxiety disorders and autism spectrum disorder (ASD) (9, 19).

Abbreviations: ASD, Autism spectrum disorder; ANS, Autonomic nervous system; FXS, Fragile X syndrome; HR, Heart rate; HRV, Heart rate variability; IBI, Interbeat interval; PNS, Parasympathetic nervous system; RSA, Respiratory sinus arrhythmia; SNS, Sympathetic nervous system.

Much of what is known about ANS function and its role in psychiatric disorders comes from studies of older TD children and adults; however, emerging evidence from longitudinal studies has provided some insight on trajectories of ANS function across early neurotypical development. For example, Bar-Haim et al. (20) examined stability and developmental change in baseline IBI and HRV across the first four years of life in TD children. Interbeat interval was shown to increase steadily from four months to four years, with the greatest increase between four and nine months. Heart rate variability also increased steadily across this age range. In a series of longitudinal studies, Patriquin et al. (21, 22) characterized baseline RSA across the first four years of life in relation to a variety of social-emotional and behavioral symptoms at age four. Results revealed that developmental trajectories of baseline RSA could be best characterized by two patterns, a "typical" pattern, in which RSA increased steadily across development, and an "atypical" pattern, in which RSA was consistently lower or plateaued over time. Of relevance to the current study, the children with atypical RSA trajectories exhibited more behavioral problems (e.g., withdrawal, aggression, oppositional defiance), and poorer social responsiveness, and all children with parent-reported developmental delay or ASD exhibited atypical RSA growth trajectories (21, 22).

According to Porges' polyvagal theory (3), healthy ANS function, driven primarily by PNS-mediated vagal regulation of the heart, supports social engagement and social learning. Given that ASD is characterized primarily by social and social-communicative impairments, much prior research has focused on non-syndromic ASD, and ANS dysfunction in individuals with non-syndromic ASD is fairly well established. Most studies have shown that children with ASD exhibit elevated HR and lower HRV at baseline, suggesting attenuated PNS function and subsequent chronic hyperarousal, as well as atypical reactivity to stressors [for thorough reviews, see (19, 23–25). In children with ASD, ANS dysfunction has been linked with poorer social functioning, higher sensory sensitivity, greater anxiety, and poorer emotion recognition (19, 21, 23, 26–30). Furthermore, recent work has suggested that reduced SNS activity differentiates

children with ASD with and without anxiety, whereas reduced PNS activity differentiates children with ASD from typically developing children (31). In the only longitudinal study of baseline ANS function in ASD, Sheinkopf and colleagues (32) found that children with ASD exhibited less change in RSA across early childhood, with significantly lower RSA evident as early as 18 months, while HR developed similarly in typically developing (TD) children and children with ASD. However, this study focused on infants at high risk for ASD because of prenatal drug exposure, with only 12 children with ASD in the sample. Thus, it is difficult to generalize these findings or draw conclusions about how ANS function develops across infancy and early childhood in the majority of children with ASD.

It has been hypothesized that ANS dysfunction plays a significant role in anxiety disorders as well (33, 34). For example, in the autonomic inflexibility model, Friedman (33) posits that biological competence is characterized by strong vagal regulation (i.e., high HRV), which consequently permits adaptive physiological and behavioral response to stress or fear followed by rapid physiological recovery. When vagal regulation is reduced or disrupted, as reflected by lower HRV, physiological responding becomes chronically blunted, rigid, and predictable, and physiological recovery is slower, ultimately leading to the emergence and maintenance of anxious symptoms. In support of this theory, lower baseline HRV has been associated with greater anxiety symptoms in children (35), and children with anxiety disorders exhibit lower baseline HRV than those without (36, 37). Several studies have also reported elevated baseline HR in children with social anxiety symptoms, suggesting that chronic hyperarousal plays a role in social anxiety (37–39). To our knowledge, no studies have examined how ANS maturation across early development relates to later anxiety symptoms in either typical development or neurodevelopmental disorders.

ANS dysfunction has also been implicated in fragile X syndrome (FXS), a monogenic disorder cause by a CGG trinucleotide expansion mutation on the *FMR1* gene on the X chromosome. Fragile X syndrome is relatively rare, affecting approximately 1 in 7,000 males and 1 in 11,000 females (40). It is characterized by moderate intellectual disability, behavior problems, and sensory processing difficulties. Children with FXS are also at significantly increased risk for both ASD (∼61%) and anxiety (∼40–50%) (41–45), both of which are linked with poorer developmental and social outcomes (46–50). Understanding how ANS dysfunction contributes to increased risk for ASD and anxiety in infants with FXS is of critical importance, as such knowledge will enable early identification of high-risk infants and inform the development of targeted prevention and interventions, which may in turn optimize developmental outcomes and quality of life for children with FXS. Furthermore, the elevated risk for these clinical features along with relatively well understood molecular-genetic and neurobiological mechanisms make FXS an ideal model to improve our understanding of gene-behavior associations related to emerging psychopathology in childhood.

For nearly three decades, the "hyperarousal hypothesis" of FXS has argued that ANS dysfunction underpins many symptoms of FXS (51, 52). Most evidence for hyperarousal in FXS comes from cross-sectional studies of older children and adolescents, which have documented elevated HR and attenuated HRV at baseline as well as atypical reactivity to challenges (30, 53–55). However the emergence, trajectory, and consequences of ANS dysfunction in FXS have not been characterized, thus the developmental origins of ANS dysfunction in FXS remain poorly understood. Despite the limited understanding of ANS dysfunction in FXS, the assumption that "hyperarousal" is a primary causal mechanism for many of the phenotypic features in FXS remains fairly widespread. For example, according to an expert consensus document (56) published on the National Fragile X Foundation website (www.fragilex.org), "Individuals with FXS are always susceptible to hyperarousal," "…hyperarousal…is believed to underlie many of the phenotypical behaviors commonly associated with fragile X syndrome," and "When problem behaviors occur, it is important to recognize that they may be caused by hyperarousal…" However, surprisingly little research has directly examined the relationship between ANS dysfunction and the FXS behavioral phenotype, particularly using longitudinal methods in early development. Roberts et al. (57) documented elevated HR and lower RSA in the youngest sample to date (8–40 months of age), and found that lower RSA was associated with greater ASD symptoms in children aged 22 months or older, but not in younger children. In the only longitudinal study to date in FXS, Roberts et al. (41) found that shorter initial IBI in infancy, but not change over time, predicted later ASD diagnoses in young children with FXS. Interestingly, neither initial RSA nor change over time in RSA predicted ASD diagnoses. These findings are surprising given that PNS dysfunction, as indexed by HRV and RSA, is thought to support social engagement and social learning (3) and has been consistently linked to non-syndromic ASD (19, 24).

In conclusion, despite the long-standing "hyperarousal hypothesis" in FXS, most previous studies have examined ANS function in FXS cross-sectionally across a limited age range. Furthermore, there has been little emphasis on infancy, developmental change across early childhood, or the developmental consequences of early ANS dysfunction on later psychopathology. The first aim of this longitudinal study was to characterize developmental change in ANS function across infancy and early childhood in FXS and typical development using the cardiac indices of IBI and RSA. Based on previous cross-sectional work in children with FXS, we hypothesized that a profile of hyperarousal, indexed by shorter IBI and lower RSA, emerges early in life increases in severity across early development in FXS. We also hypothesized that early ANS dysfunction has important developmental repercussions, conferring heightened risk for later ASD and anxiety symptoms. Thus, the second aim of the study was to examine the relationship between early ANS function and developmental change in ANS function with later ASD and anxiety symptoms. Based on leading theories of the role of ANS dysfunction in the development of anxiety (33, 34) and ASD symptoms (3, 25), we predicted that shorter IBI and slower change in IBI across development would be related to later anxiety symptoms, while lower RSA and slower

TABLE 1 | Participant characteristics: research question 1.

	FXS ($n = 73$)	TD ($n = 79$)
Total number of observations	175	197
Observations per participant, M (SD)	2.40 (1.38)	2.49 (1.89)
Observations per participant, Range	1–6	1–7
Chronological age (months), M (SD)	33.96 (21.85)	34.10 (23.97)
Chronological age (months), Range	3.05–82.99	5.67–83.81

TABLE 2 | Participant characteristics at outcome timepoint: research question 2.

	FXS ($n = 28$)	TD ($n = 25$)	$t_{(51)}$	p-value
Chronological age (months), M (SD)	63.96 (12.04)	63.22 (12.49)	−0.22	0.828
Chronological age (months), Range	46.76–83.55	35.78–83.81		
Developmental level, M (SD)	51.39 (9.29)	102.08 (13.78)	15.85	<001
Developmental level, Range	30–76	70–130		
ASD Diagnosis, *n* (%)	20 (71.43%)	0 (0%)		
ADOS-2 CSS, M (SD)	6.07 (2.28)	1.84 (1.31)	−8.16	<001
ADOS-2 CSS, Range	1–10	1–6		
PAS total raw score, M (SD)	16.04 (9.03)	12.08 (7.41)	−1.73	0.090
PAS total raw score, Range	0–39	3–25		

change in RSA across development would be related to later ASD symptoms.

MATERIALS AND METHODS

Participants

This study included 152 male children (FXS: $n = 73$; TD: $n = 79$). Baseline heart activity was measured longitudinally between 3 and 83 months of age, resulting in 372 observations (FXS: $n = 175$; TD $n = 197$). Groups were matched on average chronological age, $t_{(370)} = 0.06$, $p = 0.954$, and exhibited similar distributions of observations across age. This age range was selected to maximize the number of longitudinal heart activity data points available per participant in order to address our first research question of characterizing the emergence and trajectory of autonomic dysfunction across infancy and early childhood (i.e., infancy through six years of age). Participant characteristics are detailed in **Table 1**. Participants were drawn from two sites that collected baseline heart activity data longitudinally across infancy and early childhood. Data for 77 children (FXS: $n = 36$; TD $n = 41$) children were collected at the University of North Carolina at Chapel Hill (P30HD003110; PI: Bailey), and data for 75 children (FXS: $n = 37$; TD $n = 38$) were collected at the University of South Carolina (R01MH107573, R01MH090194; PI: Roberts). Thus, this is a convenience sample that provides the opportunity to analyze the developmental trajectory of ANS function from a large sample of infants and children with a rare genetic disorder. As a result, the present study represents the largest longitudinal sample of males with FXS ($n = 73$) spanning the most inclusive age range across early development (3–83 months) to date. Heart activity collection and processing procedures were identical at both study sites and were designed and overseen by the senior author. Some of the data included in the present study have been included in previous cross-sectional studies (53, 58–60) and a recent longitudinal study that examined various predictors of ASD diagnoses in preschool-aged males and females with FXS (41). The objectives and analyses of the present study are quite different from those of previous studies, and no studies have been published that focus on characterizing ANS function using longitudinal data across this age range or with such a large sample.

Recruitment procedures and inclusionary criteria were similar across sites. Children with FXS were recruited through local and national organizations that serve children with FXS. Diagnosis of FXS was confirmed through genetic report (i.e., >200 CGG repeats on 5′UTR on *FMR1*). TD children were recruited through advertisements placed in the community and were required to have no family history of ASD, FXS, or related disorders. Typical development was confirmed by direct clinical-behavioral testing through the larger studies, and was defined by a standard score of ≥70 on the Mullen Scales of Early Learning [MSEL; (61)] or Differential Abilities Scale, Second Edition [DAS-II; (62)] and case review via a thorough clinical best estimate review procedure (41, 63). Participants were excluded if they were born at <37 weeks gestation, had uncorrected vision or hearing impairments, or had parents who were not proficient in English.

To investigate our second research question of whether early ANS function or developmental change in ANS function across early development predicted later ASD or anxiety symptoms, we included a subset of children from the larger sample who had at least two heart activity data points and completed clinical-behavioral "outcome" testing at ≥36 months of age (FXS: $n = 28$; TD: $n = 25$). These children were drawn exclusively from the University of South Carolina study, as that study included outcome assessments of ASD and anxiety symptoms using gold-standard diagnostic assessment procedures. The groups did not differ on chronological age at the outcome timepoint, although, as expected, they did differ on developmental level and ASD symptom severity (see **Table 2**).

Measures

Heart Activity

Heart activity data were collected during a baseline period with a telemetry-based monitor (Alive Technologies, Copyright 2005–2009; CamNtech Ltd., Cambridge, UK). Infants aged 3–30 months sat on a parent's lap or in a highchair and watched a 3-min Baby Einstein video and children aged 31–83 months sat in a chair and watched a 5-min Pixar animated short film. Both videos contained music and sound effects, but no spoken language. Interbeat interval (i.e., IBI, the time in milliseconds between two consecutive heartbeats) was visually inspected and edited to correct arrhythmias, false heart periods, and artifacts

by trained research assistants using CardioEdit software (Brain-Body Center, University of Illinois at Chicago). Heart activity data were excluded if >10% of the file was edited. In this sample, an average of 1.09% of IBI values were edited per file. Respiratory sinus arrhythmia (RSA) was extracted from the edited IBI data using CardioBatch software (Brain-Body Center, University of Illinois at Chicago). CardioBatch samples sequential heart periods at 250 ms epochs and then uses a 21-point moving polynomial algorithm to detrend the data (64). The data are bandpass filtered to extract variance associated with the spontaneous breathing frequency for infants aged 3–30 months (0.3–1.3 Hz) or for children aged 31–83 months (0.24–1.04 Hz). The variance is then transformed into its natural logarithm to provide an estimate of RSA. Mean IBI and mean RSA for the entire baseline period were computed in CardioBatch and used as dependent variables in this study.

ASD Symptoms

The Autism Diagnostic Observation Schedule, Second Edition [ADOS-2; (65)] was administered by a research-reliable examiner to assess ASD symptom severity at the outcome timepoint. The ADOS-2 is a play-based, semi-structured measure developed for children and adults older than 12 months of age. The overall Calibrated Severity Score (CSS) provided a continuous index of ASD symptom severity. Scores range from 1 to 10, with higher values reflecting more severe ASD symptoms. To investigate inter-rater agreement, 18.9% of ADOS administrations in the present study were coded by a second research-reliable examiner, and an intraclass correlation (ICC) of 0.90 on overall CSS indicated excellent agreement.

Anxiety Symptoms

Anxiety symptoms were assessed at the outcome timepoint using the total raw score from the Preschool Anxiety Scale [PAS; (66)]. The PAS is a 28-item parent-report questionnaire for preschool-aged children designed to assess symptoms of anxiety and worry. The PAS demonstrates good construct, predictive, and discriminant validity as well as satisfactory internal consistency (57). Total raw scores range from 0 to 112, with higher scores indicating more severe anxiety symptoms.

Developmental Level

Developmental level was assessed at the outcome timepoint for children ≤68 months old ($n = 33$) via the Mullen Scales of Early Learning (MSEL; 64), a developmental measure designed to assess cognitive abilities in infants and children up to 68 months of age. The early learning composite (ELC), a composite score of the Visual Reception, Fine Motor, Expressive Language, and Receptive Language subscales, was used. The Differential Abilities Scale, Second Edition – [DAS-II; (62)] was used to assess developmental level in children >68 months old ($n = 20$). The DAS-II is designed to measure cognitive ability in children between 2 years, 6 months and 17 years, 11 months. Developmental level was estimated via the General Conceptual Ability (GCA) score, which is a composite of the Verbal Reasoning, Nonverbal Reasoning, and Spatial Abilities subscales.

Procedure

Procedures were approved by the Institutional Review Boards at both sites (University of North Carolina at Chapel Hill: #05-0106, initial date of approval 3/17/2003; University of South Carolina: #00001966, initial date of approval 4/30/2009). Informed consent was obtained from the parents before study enrollment. Assessments occurred at participants' homes or in research laboratories. Baseline ECG was collected at the beginning of the assessment. Parents received monetary compensation for their participation.

Statistical Analyses

Developmental change in IBI and RSA across early childhood was characterized using separate hierarchical linear models (HLMs) via SAS PROC MIXED in SAS 9.4 (SAS Institute Inc., Cary, NC). A model-building approach was utilized to determine the most parsimonious, best-fitting model (67). The results of all models considered are detailed in the **Supplementary Materials**. For both IBI and RSA, the best fitting model included the fixed and random effects of age (grand-mean centered at 34.04 months), fixed effects of group and site and the age by group interaction. Importantly, the model that included the age by group by site interaction term was a poorer fit and the interaction was non-significant, indicating that group differences in developmental change in RSA and IBI were similar across both sites.

In our second research question, which investigated whether early ANS function or developmental change in ANS function across early development predicted ASD or anxiety symptoms, the model was recentered at 24 months and slope and intercept values were extracted for each participant. This age was selected to represent a timepoint earlier in development that could be used to predict ASD and anxiety symptoms measured at ≥36 months, when ASD symptoms are considered reliable and stable and anxiety symptoms have begun to emerge. Four separate multivariate general linear models were employed in SPSS 27 (SPSS Inc., Chicago, IL) to examine how IBI intercept, IBI slope, RSA intercept, and RSA slope predicted ASD symptoms (i.e., ADOS-2 CSS score) and anxiety symptoms (i.e., PAS total raw score). Group was entered as a fixed factor and the group by heart activity variable (e.g., group by IBI intercept) was included initially to determine whether heart activity predicted symptoms differently between groups. The interaction term was non-significant in every model, so results from main effects models are reported in text. Results of non-significant interaction models are included in the **Supplementary Materials**. Developmental level was included as a covariate in these models, as it differed between groups and was correlated with RSA slope, $r = .29$, $p = 0.026$, and ASD symptom severity, $r = -0.69$, $p < 0.001$, and was marginally correlated with RSA intercept, $r = 0.26$, $p = 0.053$.

RESULTS

Change in ANS Function Across Early Development

IBI

Figure 1 depicts developmental change in IBI and RSA across age within each participant. Significant main effects of group,

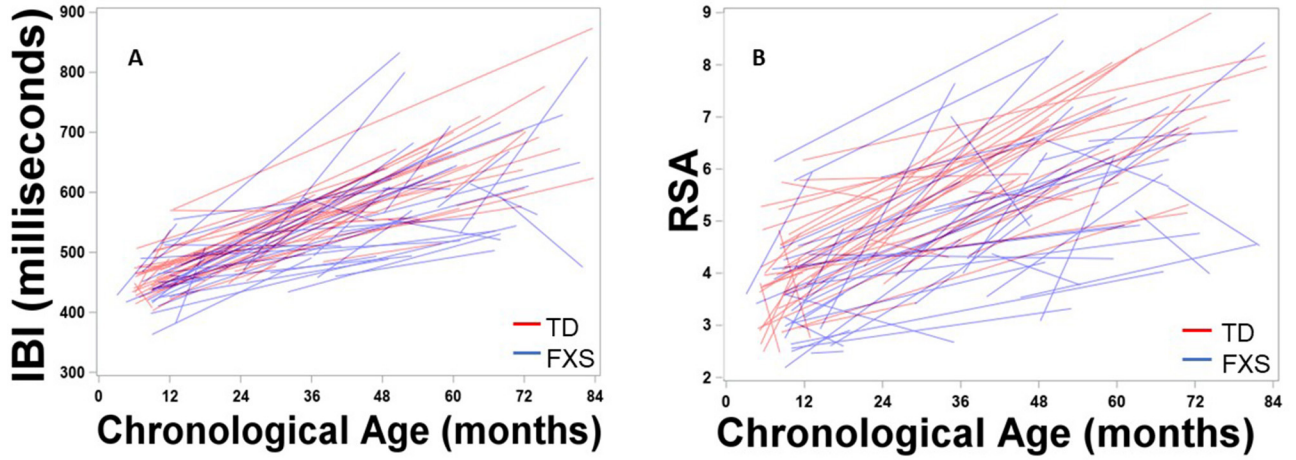

FIGURE 1 | Maturation of **(A)** IBI and **(B)** RSA across age within each participant. Males with FXS are indicated in blue; TD males are indicated in red.

TABLE 3 | Estimates for Hierarchical Linear Models of IBI and RSA across early development.

	IBI	RSA
Fixed effects		
Intercept	512.06*** (7.32)	4.72*** (0.15)
Age	3.66*** (0.24)	0.05*** (0.00)
Group (FXS)	−21.75** (7.40)	−0.54*** (0.16)
Age*Group (FXS)	−1.10** (0.36)	−0.02*** (0.01)
Site (USC)	45.88*** (7.78)	0.70*** (0.16)
Error variance		
Intercept	682.16*** (218.82)	00.47*** (0.10)
Slope	1.68*** (0.49)	0.00‡ (0.00)
ICC (from Model 1)	0.14	0.31

‡$p < 0.10$; **$p < 0.01$; ***$p < 0.001$; $n = 372$; *Values represent parameter estimates with standard errors in parentheses; Estimation method = Full; Covariance structure = Variance components.*

$F_{(1,132)} = 8.65$, $p = 0.004$, age, $F_{(1,111)} = 313.11$, $p < .001$, and site, $F_{(1,163)} = 34.77$, $p < 0.001$, were observed. A significant group by age interaction was observed, $F_{(1,116)} = 9.25$, $p = 0.003$, with IBI increasing more each month in TD children than in FXS, $b = 1.10$ms (**Table 3**, **Figure 2**). The FXS group exhibited significantly shorter IBI by 29 months, $t_{(139)} = -2.12$, $p = 0.036$.

RSA

Results indicated significant main effects of group, $F_{(1,150)} = 11.88$, $p < 0.001$, age, $F_{(1,109)} = 268.19$, $p < 0.001$, and site, $F_{(1,172)} = 18.94$, $p < 0.001$. A significant group by age interaction was observed, $F_{(1,109)} = 12.01$, $p < 0.001$, suggesting that RSA increased more each month in TD children than in FXS children, $b = 0.02$ (**Table 3**, **Figure 2**). The FXS group exhibited significantly lower RSA by 24 months, $t_{(150)} = -2.10$, $p = 0.037$.

Relationship Between ANS Function at 24 Months and ASD and Anxiety Symptoms at ≥36 Months

IBI

Full multivariate general linear model results are outlined in **Table 4**. IBI intercept was a marginally significant predictor in the overall model, $F_{(2,48)} = 2.78$, $p = 0.072$. The main effect of group was significant, $F_{(2,48)} = 4.28$, $p = 0.019$. Tests of between-subjects effects indicated that IBI intercept predicted anxiety symptoms, $F_{(1,49)} = 4.59$, $p = 0.037$, but not ASD symptoms, $F_{(1,49)} = 0.67$, $p = 0.419$ (**Figures 3A,B**). Group predicted ASD symptoms, $F_{(1,49)} = 6.58$, $p = 0.013$, but not anxiety symptoms, $F_{(1,49)} = 1.45$, $p = 0.235$. Parameter estimates suggested that for every 1ms increase in IBI intercept (i.e., IBI at 24 months), anxiety scores decreased by 0.12 points. The effect of IBI slope emerged as a significant predictor in the model, $F_{(2,48)} = 4.05$, $p = 0.024$, as did group, $F_{(2,48)} = 4.96$, $p = 0.011$. Between-subjects tests indicated that IBI slope predicted anxiety, $F_{(1,49)} = 6.75$, $p = 0.012$, but not ASD symptoms, $F_{(1,49)} = 0.85$, $p = 0.362$ (**Figures 3C,D**). Group predicted ASD symptoms, $F_{(1,49)} = 7.04$, $p = 0.011$, but not anxiety symptoms, $F_{(1,49)} = 2.06$, $p = 0.158$. Parameter estimates indicated that for every 1ms increase per month in IBI, anxiety scores decreased by 2.60 points.

RSA

RSA intercept emerged as a marginally significant overall predictor, $F_{(2,48)} = 3.10$, $p = 0.054$ (**Table 5**). The main effect of group was significant, $F_{(2,48)} = 4.43$, $p = 0.0174$. Tests of between-subjects effects indicated that RSA intercept predicted ASD symptoms, $F_{(1,49)} = 6.13$, $p = 0.017$, but not anxiety symptoms, $F_{(1,49)} = 0.06$, $p = 0.802$. Group predicted ASD symptoms, $F_{(1,49)} = 6.79$, $p = 0.012$, but not anxiety symptoms, $F_{(1,49)} = 1.66$, $p = 0.203$ (**Figures 3E,F**). Parameter estimates suggested that for every 1-unit increase in RSA at 24 months, ASD severity scores decreased by 0.91 points (**Figure 3E**). RSA Slope was not a significant predictor in the overall model, $F_{(2,48)}$

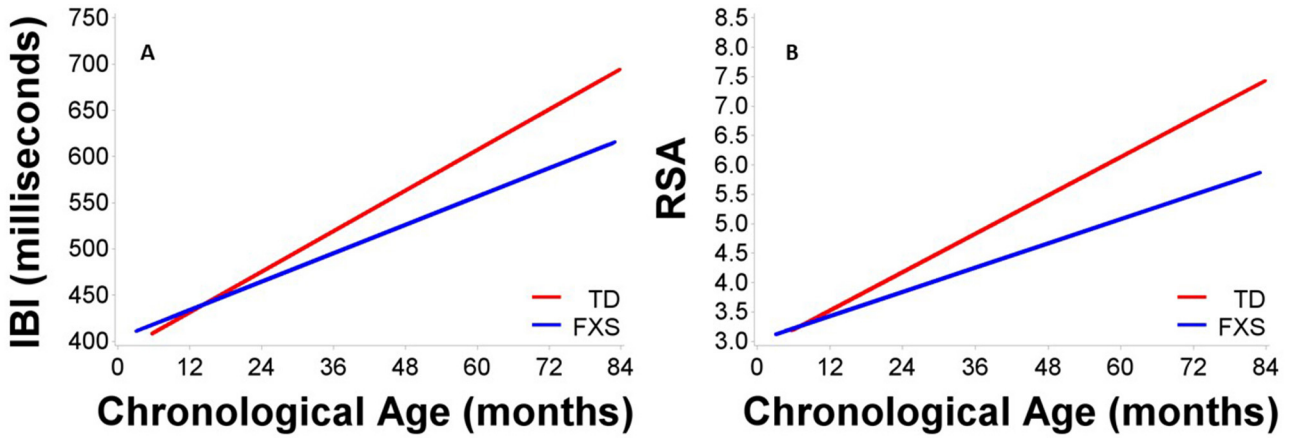

FIGURE 2 | Maturation in **(A)** IBI and **(B)** RSA across age in males with FXS and TD males. Males with FXS are indicated in blue; TD males are indicated in red.

TABLE 4 | Multivariate general linear model results for IBI intercept and IBI slope at 24 months predicting later ASD and anxiety symptoms.

	IBI Intercept				IBI Slope			
	λ	$F_{(2,48)}$	*p*-value	η_p^2	λ	$F_{(2,48)}$	*p*-value	η_p^2
Intercept	0.85	4.30	0.019	0.15	0.76	7.75	0.001	0.24
IBI	0.90	2.78	0.072	0.10	0.86	4.05	0.024	0.14
Group	0.85	4.28	0.019	0.15	0.83	4.96	0.011	0.17
Developmental Level	0.98	0.48	0.624	0.02	0.98	0.53	0.592	0.02

λ = Wilks' lambda; η_p^2 = partial eta squared.

$= 1.86$, $p = 0.166$ (**Figures 3G,H**), while group was, $F_{(2,48)} = 4.23$, $p = 0.020$. Group predicted ASD symptoms, $F_{(1,49)} = 6.45$, $p = 0.014$, but not anxiety symptoms, $F_{(1,49)} = 1.48$, $p = 0.229$.

DISCUSSION

Findings from the present study indicate that physiological hyperarousal is present by 24 months of age in males with FXS and that trajectories of hyperarousal across infancy to early childhood differentially predict ASD and anxiety symptoms. This study is the largest longitudinal study to document the developmental emergence of hyperarousal in very young children with FXS, and it provides important evidence in support of the "hyperarousal hypothesis" in FXS (51). While previous cross-sectional studies (30, 53–55, 57, 68) were critical in establishing evidence of atypical ANS function in FXS, until now the lack of dense longitudinal sampling early in development has precluded conclusions about the developmental emergence of the hyperarousal profile so often considered to be a primary source of impairments in FXS [e.g., (56)]. The emergence of hyperarousal early in development, as observed in the present study, has important long-term developmental repercussions as the first years of life represent a developmental period where multiple critical social, emotional, regulatory, and communication skills are rapidly developing alongside extensive brain maturation in a dynamic inter-dependent manner [e.g., (69)].

We were somewhat surprised that the FXS group was not statistically distinguishable from the TD group on cardiac indices earlier in infancy, given that other FXS-related developmental differences are evident earlier in development. While ANS dysfunction may originate earlier in infancy or even in prenatal development, it is possible that our relatively small number of datapoints under 12 months of age ($n = 26$), with the median age of first datapoint of 21 months, limited our statistical ability to identify differences in ANS function in infancy. It is worth noting that marginally significant group differences began to emerge at 27 months for IBI and 21 months for RSA, suggesting that perhaps with additional longitudinal data in infancy, we may have been able to detect group differences earlier in development. However, with more than half of participants ($n = 82$, 53.95%) entering the study before their second birthday (FXS: 53.42%; TD: 54.47%), our sample still represents the largest study to date of ANS function in early development in FXS. Future studies should consider denser longitudinal sampling throughout the first year of life to document the earliest potential onset and trajectory of ANS dysfunction in FXS. Understanding the timing and nature of ANS dysfunction as early as possible in infancy will provide critical opportunities for early identification, prevention, and intervention.

The relationship between early developmental trajectories of hyperarousal to increased psychiatric symptom severity later in development support the critical role that ANS function has

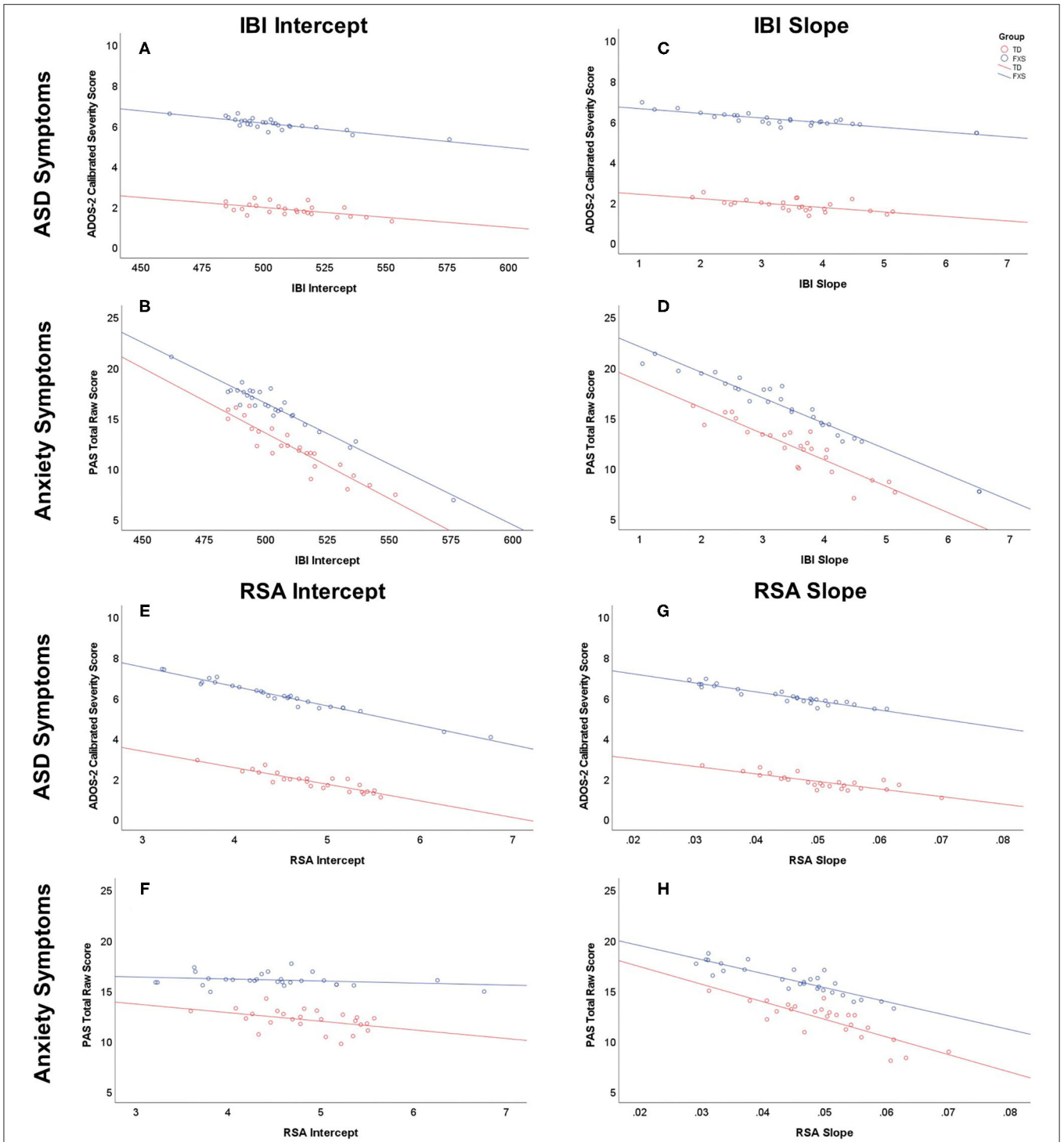

FIGURE 3 | Relationship between IBI and RSA at 24 months of age and ASD and anxiety symptoms at 36 months of age or older (predicted values). Males with FXS are indicated in blue; TD males are indicated in red. IBI intercept predicting ASD and anxiety symptoms is depicted in **(A)** and **(B)**, respectively. IBI slope predicting ASD and anxiety symptoms is depicted in **(C)** and **(D)**, respectively. RSA intercept predicting ASD and anxiety symptoms is depicted in **(E)** and **(F)**, respectively. RSA slope predicting ASD and anxiety symptoms is depicted in **(G)** and **(H)**, respectively.

on the FXS phenotype, which is characterized by high rates of both anxiety and ASD. Specifically, shorter IBI at 24 months and slower change in IBI across development predicted higher anxiety symptoms, but not ASD symptoms. The relationship between early hyperarousal and later anxiety symptoms is in line with the theory of "autonomic inflexibility" (33, 34) and empirical

TABLE 5 | Multivariate general linear model results for RSA intercept and RSA slope at 24 months predicting later ASD and anxiety symptoms.

	RSA intercept				RSA slope			
	λ	$F_{(2,48)}$	*p*-value	η_p^2	λ	$F_{(2,48)}$	*p*-value	η_p^2
Intercept	0.74	8.57	0.001	0.26	0.77	7.09	0.002	0.23
RSA	0.89	3.10	0.054	0.12	0.93	1.86	0.166	0.07
Group	0.84	4.43	0.017	0.16	0.85	4.23	0.020	0.15
Developmental Level	0.98	0.42	0.658	0.02	0.98	0.46	0.635	0.02

λ = *Wilks' lambda;* η_p^2 = *partial eta squared.*

work that has documented the relationship between elevated baseline arousal and anxiety in neurotypical children (37–39). Generally, these studies suggest that anxiety is characterized by heightened arousal and reduced parasympathetic activity at baseline as well as blunted reactivity to and delayed recovery from stress. While our study did not investigate autonomic reactivity or recovery, previous cross-sectional studies of autonomic reactivity to social stress in FXS have yielded conflicting results that are suggestive of developmental shifts in both baseline activity and autonomic reactivity in FXS across infancy and early childhood. For example, 12-month-old infants with FXS have been shown to exhibit normative baseline RSA but reduced RSA reactivity to a stranger approach (60), whereas young children with FXS exhibit shorter IBI and lower RSA at baseline but typical patterns of reactivity and recovery when approached by a stranger (58). Interestingly, shorter IBI was associated with more behavioral indicators of fear in younger children (<29 months), while longer IBI was associated with more behavioral indicators of fear in older children (>51 months). These findings, taken together with the findings of the present study, highlight the importance of longitudinal work in elucidating the developmental emergence and developmental trajectory of autonomic dysfunction in FXS. Furthermore, while our findings suggest a relationship between developmental change in baseline hyperarousal and later anxiety symptoms, much remains to be understood about the role that autonomic reactivity to and recovery from stress plays in the development of anxiety symptoms in children with FXS.

In the present study, lower RSA at 24 months predicted greater ASD symptoms, but not anxiety symptoms. Our findings of a relationship between lower baseline RSA and higher ASD symptoms parallel converging evidence of poor parasympathetic regulation in children with non-syndromic ASD [for thorough reviews, see (19, 23–25)]. These findings, in combination with our own that document a relationship between early RSA, but not early IBI, and later ASD symptoms, suggest that parasympathetic activity may play an important role in ASD, whereas overall arousal may be less relevant. This is in line with leading theories (e.g., polyvagal theory) that argue that PNS-mediated regulation of the vagal nerve supports social engagement, social learning, and social-emotional development (3). Our findings also correspond with recent research that suggests that lower parasympathetic activity can differentiate children with ASD from TD controls but does not differentiate anxious and non-anxious children with ASD (31). Previous studies of FXS have also suggested that parasympathetic activity may also predict ASD symptoms, but the findings have been nuanced. For example, IBI has been shown to predict later ASD symptoms in a non-linear fashion across age, with greater ASD severity in childhood being associated with longer IBI in infancy but shorter IBI in childhood (57). In the only longitudinal study of ANS function in young children with FXS to date, Roberts and colleagues (41) found that shorter initial IBI, but not developmental change in IBI, predicted later ASD diagnoses in young children with FXS. Interestingly, neither initial RSA nor RSA maturation predicted ASD diagnoses. It is important to note that some data from these previous studies are included in the convenience sample used in the present study, but there are several important methodological differences that may explain why our findings differed. For example, Roberts and colleagues (41) examined categorical diagnostic outcomes while we used a continuous measure of ASD symptom severity which may have provided more variability and statistical power. Both of these previous studies also included females in their study, while the present study was limited to males, who tend to be more severely impaired and have higher ASD symptom severity (70).

Surprisingly, developmental change in RSA did not predict ASD symptoms later in childhood, despite findings of developmental change in RSA being linked with later ASD symptoms in previous studies of low-risk and high-risk infants (21, 22, 32). For example, slower developmental change in RSA has been linked with later ASD diagnoses in infants with prenatal drug exposure (32). Slower or plateauing developmental change in RSA has also been linked with behaviors associated with ASD, such as behavioral problems (e.g., withdrawal, aggression, oppositional defiance) and poorer social responsiveness (21, 22). The findings of the present study suggest that it is RSA in early development, not growth or change over time, that has long-term impact on ASD symptoms.

Limitations and Future Directions

This study is the largest longitudinal study of ANS function in FXS to date, and it is the first to examine developmental emergence and change in ANS function across infancy and early childhood in relation to symptoms of anxiety of ASD, which are the most common psychiatric comorbidities of FXS. Despite our important findings, certain limitations should be considered. First, the nature of our convenience sample compiled from multiple larger studies was both a strength, in that

it enabled us to examine ANS function in a large sample of infants and children with a rare genetic disorder, and a weakness, in that some measures (e.g., outcome measures) were available in only a subset of participants. Additionally, we did not have detailed molecular-genetic data (e.g., FMRP, methylation) on most of our participants, so we were unable to investigate the relationship between molecular-genetic variation and physiological hyperarousal. These analyses would provide valuable information about the pathophysiological mechanisms underlying atypical development in FXS and should be considered in future studies.

Examining early development over a large age range also posed certain methodological challenges, including discontinuity of measures across development. For example, in our study, the Mullen Scales of Early Learning (61) was used as a measure of developmental level through early childhood, but it is only appropriate until 68 months of age, at which point we used the Differential Abilities Scale–Second Edition [DAS-II; (62)]. However, because identical procedures were used in both FXS and TD, and developmental level served as a covariate at the outcome timepoint and not a primary variable of interest, we believe this is a minor limitation. Finally, this study was limited to males, and the exclusion of females with FXS restricts the generalizability of findings to all children with FXS. Because of their unique clinical profiles and the fact that FXS is less common in females (40), females have often been overlooked in previous studies (71). Work focused on characterizing and understanding ANS function in females with FXS is desperately needed, and we intend to address that critical gap in the future.

Summary and Implications

Findings indicate that ANS dysfunction and hyperarousal emerge by 24 months of age in males with FXS. Importantly, hyperarousal across early childhood predicts elevated symptom severity for both anxiety and ASD, which is in line with the "hyperarousal hypothesis" of FXS and clearly documented negative impact of hyperarousal on multiple domains of function in non-syndromic populations. The findings of the present study have several important clinical implications. For example, diagnostic efforts should account for the potential impact of hyperarousal on the process and outcome of diagnostic differentiation in children with FXS. Clinicians should consider the role that hyperarousal may be playing in behaviors observed during diagnostic assessments and those reported by parents. Measurement of hyperarousal during the diagnostic process may provide complementary insight into risk for anxiety or ASD symptoms. Additionally, treatment efforts should consider that physiological hyperarousal might represent a modifiable factor that could increase behavioral intervention efficacy. For example, learning to recognize the signs of hyperarousal, directly monitoring arousal in real time, and incorporating calming activities into treatment as needed might be effective approaches to maximizing treatment benefit.

Given that ANS dysfunction, and in particular, attenuated PNS function, has been shown to impact social-communicative development specifically (3), hyperarousal in infancy and early childhood may have detrimental long-term impacts on brain development and social-communicative functioning. However, research on non-syndromic ASD has clearly documented that this developmental period is a prime period for maximum impact of targeted early intervention (72, 73). Furthermore, ANS dysfunction may be an especially promising target for early intervention in FXS, as several treatment methods have been shown to be effective in improving ANS dysfunction and ANS-related symptoms in other disorders. For example, non-invasive transcutaneous vagal nerve stimulation has been shown to be effective in reducing hyperarousal in veterans with posttraumatic stress disorder and traumatic brain injury (74). It is also effective in reducing severity of drug-resistant epilepsy (75) and major depressive disorder (76, 77), and it has been proposed as a potential treatment in non-syndromic ASD (78). Additionally, HRV biofeedback has been shown to be effective in increasing HRV and decreasing symptoms of depression, anxiety, and insomnia in patients with major depressive disorder (79–82), though the cognitive demands of a biofeedback protocol may be difficult for some children with FXS. The findings of the present study suggest that hyperarousal is present very early in development in FXS and has detrimental long-term consequences. Thus, future studies should investigate the efficacy of treatment approaches that directly target ANS dysfunction in FXS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of South Carolina Institutional Review Board and University of North Carolina at Chapel Hill Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

AH conceived of study questions, analyzed the data, compiled results, and created figures. AH drafted the manuscript with substantive contributions from EH, KS, CB, and KB. KS oversaw processing of heart activity data. JK and JR aided in interpretation of results and refinement of the manuscript. JR obtained study funding. All authors discussed the results and commented on the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.727559/full#supplementary-material

REFERENCES

1. Thayer JF, Lane RD. A model of neurovisceral integration in emotion regulation and dysregulation. *J Affect Disord.* (2000) 61:201–16. doi: 10.1016/s0165-0327(00)00338-4
2. Beauchaine TP. Vagal tone, development, and gray's motivational theory: toward an integrated model of autonomie nervous system functioning in psychopathology. *Dev Psychopathol.* (2001) 13:183–214. doi: 10.1017/s0954579401002012
3. Porges SW. The polyvagal theory: phylogenetic substrats of a social nervous system. *Int J Psychophysiol.* (2001) 42:123–46. doi: 10.1016/s0167-8760(01)00162-3
4. Porges SW. The polyvagal perspective. *Biol Psychol.* (2007) 74:116–43. doi: 10.1016/j.biopsycho.2006.06.009
5. Thayer JF, Brosschot JF. Psychosomatics and psychopathology: looking up and down from the brain. *Psychoneuroendocrinology.* (2005) 30:1050–8. doi: 10.1016/j.psyneuen.2005.04.014
6. Levy MN. Cardiac sympathetic-parasympathetic interactions. *Fed Proc.* (1984) 43:2598–602.
7. Bernston GG, Bigger TJ, Eckberg DL, Grossman P, Kaufmann PG, Malik M, et al. Heart rate variability: origins, methods, and interpretive caveats. *Psychophysiology.* (1997) 34:623–48. doi: 10.1111/j.1469-8986.1997.tb02140.x
8. Robinson BF, Epstein SE, Beiser GD, Braunwald E. Control of heart rate by the autonomic nervous system. Studies in man on the interrelation between baroreceptor mechanisms and exercise. *Circ Res.* (1966) 19:400–11. doi: 10.1161/01.res.19.2.400
9. Beauchaine TP. Elsevier. Respiratory sinus arrhythmia: a transdiagnostic biomarker of emotion dysregulation and psychopathology. *Curr Opin Psychol.* (2015) 3:43–7. doi: 10.1016/j.copsyc.2015.01.017
10. Bernston GG, Cacioppo JT, Quigley K. Respiratory sinus arrhythmia: autonomic origins, physiological mechanisms, and psychophysiological implications. *Psychophysiology.* (1993) 30:183–96. doi: 10.1111/j.1469-8986.1993.tb01731.x
11. Porges SW. Cardic vagal tone: a physiological index of stress. *Neurosci Biobehav Rev.* (1995) 19:225–33. doi: 10.1016/0149-7634(94)00066-a
12. Thayer JF, Siegle GJ. Neurovisceral integration in cardiac and emotional regulation: a model of the central nervous system concomitants of heart-rate variability in humans. *IEEE Eng Med Biol Mag.* (2002) 21:24–9. doi: 10.1109/memb.2002.1032635
13. Park G, van Bavel JJ, Vasey MW, Thayer JF. Cardiac vagal tone predicts attentional engagement to and disengagement from fearful faces. *Emotion.* (2013) 13:645–56. doi: 10.1037/a0032971
14. Hansen AL, Johnsen BH, Thayer JF. Vagal influence on working memory and attention. *Int J Psychophysiol.* (2003) 48:263–74. doi: 10.1016/s0167-8760(03)00073-4
15. Park G, Van Bavel JJ, Vasey MW, Thayer JF. Cardiac vagal tone predicts inhibited attention to fearful faces. *Emotion.* (2012) 12:1292–302. doi: 10.1037/a0028528
16. Porges SW. Vagal tone: a physiologic marker of stress vulnerability. *Pediatrics.* (1992) 90(3 ii Suppl):498—504.
17. Thayer JF, Lane RD. The role of vagal function in the risk for cardiovascular disease and mortality. *Biol Psychol.* (2007) 74:224–42. doi: 10.1016/j.biopsycho.2005.11.013
18. Doussard-Roosevelt JA, Mcclenny BD, Porges SW. Neonatal cardiac vagal tone and school-age developmental outcome in very low birth weight infants. *Dev Psychobiol.* (2001) 38:56–66. doi: 10.1002/1098-2302(2001)38:1%3C56::AID-DEV5%3E3.0.CO;2-K
19. Klusek J, Roberts JE, Losh M. Cardiac autonomic regulation in autism and fragile X syndrome: a review. *Pychol Bull.* (2015) 141:141–75. doi: 10.1037/a0038237
20. Bar-Haim Y, Marshall PJ, Fox NA. Developmental changes in heart period and high-frequency heart period variability from 4 months to 4 years of age. *Dev Psychobiol.* (2000) 37:44–56. doi: 10.1002/1098-2302(200007)37:1%3C44::AID-DEV6%3E3.0.CO;2-7
21. Patriquin MA, Lorenzi J, Scarpa A, Bell MA. Developmental trajectories of respiratory sinus arrhythmia: associations with social responsiveness. *Dev Psychobiol.* (2014) 56:317–26. doi: 10.1002/dev.21100
22. Patriquin MA, Lorenzi J, Scarpa A, Calkins SD, Bell MA. Broad implications for respiratory sinus arrhythmia development: associations with childhood symptoms of psychopathology in a community sample. *Dev Psychobiol.* (2015) 57:120–30. doi: 10.1002/dev.21269
23. Patriquin MA, Hartwig EM, Friedman BH, Porges SW, Scarpa A. Autonomic response in autism spectrum disorder: relationship to social and cognitive functioning. *BiolPpsychol.* (2019) 145:185–97. doi: 10.1016/j.biopsycho.2019.05.004
24. Cheng YC, Huang YC, Huang WL. Heart rate variability in individuals with autism spectrum disorders: a meta-analysis. *Neurosci Biobehav Rev.* (2020) 118:463–71. doi: 10.1016/j.neubiorev.2020.08.007
25. Arora I, Bellato A, Ropar D, Hollis C, Groom MJ. Is autonomic function during resting-state atypical in autism: a systematic review of evidence. *Neurosci Biobehav Rev.* (2021) 125:417–41. doi: 10.1016/j.neubiorev.2021.02.041
26. Bal E, Harden E, Lamb D, van Hecke AV, Denver JW, Porges SW. Emotion recognition in children with autism spectrum disorders: relations to eye gaze and autonomic state. *J Autism Dev Disord.* (2010) 40:358–70. doi: 10.1007/s10803-009-0884-3
27. Hollocks MJ, Howlin P, Papadopoulos AS, Khondoker M, Simonoff E. Differences in HPA-axis and heart rate responsiveness to psychosocial stress in children with autism spectrum disorders with and without co-morbid anxiety. *Psychoneuroendocrinology.* (2014) 46:32–45. doi: 10.1016/j.psyneuen.2014.04.004
28. Neuhaus E, Bernier R, Beauchaine TP. Brief report: social skills, internalizing and externalizing symptoms, and respiratory sinus arrhythmia in autism. *J Autism Dev Disord.* (2014) 44:730–7. doi: 10.1007/s10803-013-1923-7
29. van Hecke AV, Lebow J, Bal E, Lamb D, Harden E, Kramer A, et al. Electroencephalogram and heart rate regulation to familiar and unfamiliar people in children with autism spectrum disorders. *Child Dev.* (2009) 80:1118–33. doi: 10.1111/j.1467-8624.2009.01320.x
30. Klusek J, Martin GE, Losh M. Physiological arousal in autism and fragile X syndrome: group comparisons and links with pragmatic language. *Am J Intellect Dev Disabil.* (2013) 118:475–95. doi: 10.1352/1944.7558-118.6.475
31. Parma V, Cellini N, Guy L, Mcvey AJ, Rump K, Worley J, et al. Profiles of autonomic activity in autism spectrum disorder with and without anxiety. *J Autism Dev Disord.* (2021) 2021:1–12. doi: 10.1007/s10803-020-04862-0
32. Sheinkopf SJ, Levine TP, Mccormick C, Puggioni G, Conradt E, Lagasse LL, et al. Developmental trajectories of autonomic functioning

in autism from birth to early childhood. *Biol Psychol.* (2019) 142:13–8. doi: 10.1016/j.biopsycho.2019.01.003
33. Friedman BH. An autonomic flexibility-neurovisceral integration model of anxiety and cardiac vagal tone. *Biol Psychol.* (2007) 74:185–99. doi: 10.1016/j.biopsycho.2005.08.009
34. Clark LA, Watson D. Tripartite model of anxiety and depression: psychometric evidence and taxonomic implications. *J Abnorm Psychol.* (1991) 100:316–36. doi: 10.1037/0021-843x.100.3.316
35. Scott BG, Weems CF. Resting vagal tone and vagal response to stress: associations with anxiety, aggression, and perceived anxiety control among youths. *Psychophysiology.* (2014) 51:718–27. doi: 10.1111/psyp.12218
36. Dieleman GC, Huizink AC, Tulen JH, Utens EM, Creemers HE, van der Ende J, et al. Alterations in hpa-axis and autonomic nervous system functioning in childhood anxiety disorders point to a chronic stress hypothesis. *Psychoneuroendocrinology.* (2015) 51:135–50. doi: 10.1016/j.psyneuen.2014.09.002
37. Schmitz J, Krämer M, Tuschen-Caffier B, Heinrichs N, Blechert J. Restricted autonomic flexibility in children with social phobia. *J Child Psychol Psychiatry Allied Discip.* (2011) 52:1203–11. doi: 10.1111/j.1469-7610.2011.02417.x
38. Krämer M, Seefeldt WL, Heinrichs N, Tuschen-Caffier B, Schmitz J, Wolf OT, et al. Subjective, autonomic, and endocrine reactivity during social stress in children with social phobia. *J Abnorm Child Psychol.* (2012) 40:95–104. doi: 10.1007/s10802-011-9548-9
39. Siess J, Blechert J, Schmitz J. Psychophysiological arousal and biased perception of bodily anxiety symptoms in socially anxious children and adolescents: a systematic review. *Eur Child Adolesc Psychiatry.* (2014) 23:127–42. doi: 10.1007/s00787-013-0443-5
40. Hunter J, Rivero-Arias O, Angelov A, Kim E, Fotheringham I, Leal J. Epidemiology of fragile x syndrome: a systematic review and meta-analysis. *Am J Med Genet Part A.* (2014) 164:1648–58. doi: 10.1002/ajmg.a.36511
41. Roberts JE, Bradshaw J, Will E, Hogan AL, Mcquillin S, Hills K. Emergence and rate of autism in fragile x syndrome across the first years of life. *Dev Psychopathol.* (2020) 32:1335–52. doi: 10.1017/s0954579420000942
42. Bailey DB, Raspa M, Olmsted M, Holiday DB. Co-occurring conditions associated with fmr1 gene variations: findings from a national parent survey. *Am J Med Genet Part A.* (2008) 146:2060–9. doi: 10.1002/ajmg.a.32439
43. Baranek GT, Roberts JE, David FJ, Sideris J, Mirrett PL, Hatton DD, et al. Developmental trajectories and correlates of sensory processing in young boys with fragile x syndrome. *Phys Occup Ther Pediatr.* (2008) 28:79–98. doi: 10.1300/j006v28n01_06
44. Cordeiro L, Ballinger E, Hagerman R, Hessl D. Clinical assessment of dsm-iv anxiety disorders in fragile x syndrome: prevalence and characterization. *J Neurodev Disord.* (2011) 3:57–67. doi: 10.1007/s11689-010-9067-y
45. Ezell J, Hogan A, Fairchild A, Hills K, Klusek J, Abbeduto L, et al. Prevalence and predictors of anxiety disorders in adolescent and adult males with autism spectrum disorder and fragile x syndrome. *J Autism Dev Disord.* (2019) 49:1131–41. doi: 10.1007/s10803-018-3804-6
46. Chang Y-C, Quan J, Wood JJ. Effects of anxiety disorder severity on social functioning in children with autism spectrum disorders. *J Dev Phys Disabil.* (2012) 24:235–45. doi: 10.1007/s10882-012-9268-2
47. Will EA, Bishop SL, Roberts JE. Developmental divergence: motor trajectories in children with fragile x syndrome with and without co-occurring autism. *J Neurodev Disord.* (2019) 11:23. doi: 10.1186/s11689-019-9281-1
48. Bailey DB, Hatton DD, Skinner M, Mesibov G. Autistic behavior, fmr1 protein, and developmental trajectories in young males with fragile x syndrome. *J Autism Dev Disord.* (2001) 31:165–74. doi: 10.1023/a:1010747131386
49. Talisa VB, Boyle L, Crafa D, Kaufmann WE. Autism and anxiety in males with fragile x syndrome: an exploratory analysis of neurobehavioral profiles from a parent survey. *Am J Med Genet Part A.* (2014) 164:1198–203. doi: 10.1002/ajmg.a.36468
50. Thurman AJ, Mcduffie A, Hagerman R, Abbeduto L. Psychiatric symptoms in boys with fragile X syndrome: a comparison with nonsyndromic autism spectrum disorder. *Res Dev Disabil.* (2014) 35:1072–86. doi: 10.1016/j.ridd.2014.01.032
51. Cohen IL. A theoretical analysis of the role of hyperarousal in the learning and behavior of fragile X males. *Ment Retard Dev Disabil Res Rev.* (1995) 1:286–91. doi: 10.1002/mrdd.1410010410
52. Belser RC, Sudhalter V. Arousal difficulties in males with fragile x syndrome: a preliminary report. *Dev Brain Dysfunct.* (1995) 8:270–9.
53. Roberts JE, Boccia ML, Bailey DB, Hatton DD, Skinner M. Cardiovascular indices of physiological arousal in boys with fragile X syndrome. *Dev Psychobiol.* (2001) 39:107–23. doi: 10.1002/dev.1035
54. Heilman KJ, Harden ER, Zageris DM, Berry-Kravis E, Porges SW. Autonomic regulation in fragile X syndrome. *Dev Psychobiol.* (2011) 53:785–95. doi: 10.1002/dev.20551
55. Hall SS, Lightbody AA, Huffman LC, Lazzeroni LC. Reiss AL. Physiological correlates of social avoidance behavior in children and adolsecents with fragile X syndrome. *J Am Acad Child Adolesc Psychiatry*. (2009) 48:320–9. doi: 10.1097/chi.0b013e318195bd15
56. Fragile X Clinical and Research Consortium. *Consensus of the Fragile X Clinical & Research Consortium on Clinical Practices: Hyperarousal in Fragile X Syndrome*. (2012). Available online at: https://fragilex.org/wp-content/uploads/2012/08/Hyperarousal-in-Fragile-X-Syndrome2012-Oct.pdf (accessed May 12, 2021).
57. Roberts JE, Tonnsen BL, Robinson A, Shinkareva SV. Heart activity and autistic behavior in infants and toddlers with fragile X syndrome. *Am J Intellect Dev Disabil.* (2012) 117:90–102. doi: 10.1352/1944-7558-117.2.90
58. Tonnsen BL, Shinkareva SV, Deal SC, Hatton DD, Roberts JE. Biobehavioral indicators of social fear in young children with fragile X syndrome. *Am J Intellect Dev Disabil.* (2013) 118:447–59. doi: 10.1352/1944-7558-118.6.447
59. Roberts JE, Long ACJ, Mccary LM, Quady AN, Rose BS, Widrick D, et al. Cardiovascular and behavioral response to auditory stimuli in boys with fragile X syndrome. *J Pediatr Psychol.* (2013) 38:276–84. doi: 10.1093/jpepsy/jss114
60. Black CJ, Hogan AL, Smith KD, Roberts JE. Early behavioral and physiological markers of social anxiety in infants with fragile X syndrome. *J Neurodev Disord.* (2021) 13:11. doi: 10.1186/s11689-021-09356-3
61. Mullen E. *Mullen Scales of Early Learning.* Circle Pines, MN: American Guidance Service. (1995).
62. Elliott CD. *Differential Abilities Scales-II*. Bloomington, MN: Pearson Assessments. (2006).
63. Hogan AL, Caravella KE, Ezell J, Rague L, Hills K, Roberts JE. Autism spectrum disorder symptoms in infants with fragile X syndrome: a prospective case series. *J Autism Dev Disord*. (2017) 47:1628–44. doi: 10.1007/s10803-017-3081-9
64. Porges SW, Bohrer RE. The analysis of periodic processes in psychophysiological research. In: Cacioppo JT, Tassinary LG, editors. *Principles of Psychophysiology: Physical, Social, and Inferential Elements*. New York, NY: Cambridge University Press (1990). p. 708–53.
65. Lord C, Rutter M, Dilavore P, Risi S, Gotham K, Bishop S. *Autism Diagnostic Observation Schedule, Second Edition: ADOS-2.* Torrance, CA: Western Psychological Services. (2012).
66. Spence Sh, Rapee R, Mcdonald C, Ingram M. The structure of anxiety symptoms among preschoolers. *Behav Res Ther.* (2001) 39:1293–316. doi: 10.1016/s0005-7967(00)00098-x
67. Bell BA, Ene M, Smiley W, Schoeneberger JA A multilevel model primer using sas proc mixed. *Sas Glob Forum.* (2013) 433:1–19. Available online at: https://support.sas.com/resources/papers/proceedings13/433-2013.pdf
68. Boccia ML, Roberts JE. Behavior and autonomic nervous system function as assessed via heart activity: the case of hyperarousal in boys with fragile X syndrome. *Behav Res Methods Instrum Comput.* (2000) 32:5–10. doi: 10.3758/bf03200783
69. Remer J, Croteau-Chonka E, Dean Dc, D'arpino S, Dirks H, Whiley D, et al. Quantifying cortical development in typically developing toddlers and young children, 1–6 years of age. *Neuroimage*. (2017) 153:246–61. doi: 10.1016/j.neuroimage.2017.04.010
70. Clifford S, Dissanayake C, Bui QM, Huggins R, Taylor AK, Loesch DZ. Autism spectrum phenotype in males and females with fragile x full mutation and premutation. *J Autism Dev Disord.* (2007) 37:738–47. doi: 10.1007/s10803-006-0205-z
71. Bartholomay K, Lee C, Bruno J, Lightbody A, Reiss A. Closing the gender gap in fragile X syndrome: review of females with fragile X syndrome and preliminary research findings. *Brain Sci.* (2019) 9:11. doi: 10.3390/brainsci9010011

72. Granpeesheh D, Dixon DR, Tarbox J, Kaplan AM, Wilke AE. The effects of age and treatment intensity on behavioral intervention outcomes for children with autism spectrum disorders. *Res Autism Spectr Disord*. (2009) 3:1014–22. doi: 10.1016/j.rasd.2009.06.007
73. Fuller EA, Kaiser AP. The effects of early intervention on social communication outcomes for children with autism spectrum disorder: a meta-analysis. *J Autism Dev Disord*. (2020) 50:1683–700. doi: 10.1007/s10803-019-03927-z
74. Lamb DG, Porges EC, Lewis GF, Williamson JB. Non-invasive vagal nerve stimulation effects on hyperarousal and autonomic state in patients with posttraumatic stress disorder and history of mild traumatic brain injury: preliminary evidence. *Front Med*. (2017) 4:124. doi: 10.3389/fmed.2017.00124
75. Barbella G, Cocco I, Freri E, Marotta G, Visani E, Franceschetti S, et al. Transcutaneous vagal nerve stimulatio (t-VNS): an adjunctive treatment option for refractory epilepsy. *Seizure*. (2018) 60:115–9. doi: 10.1016/j.seizure.2018.06.016
76. Carreno FR, Frazer A. Vagal nerve stimulation for treatment-resistant depression. *Neurotherapeutics*. (2017) 14:716–27. doi: 10.1007/s13311-017-0537-8
77. Evensen K, Jørgensen MB, Sabers A, Martiny K. Transcutaneous vagal nerve stimulation in treatment-resistant depression: a feasibility study. *Neuromodulation Technol Neural Interface*. (2021) ner.13366. doi: 10.1111/ner.13366. [Epub ahead of print].
78. Jin Y, Kong J. Transcutaneous vagus nerve stimulation: a promising method for treatment of autism spectrum disorders. *Front Neurosci*. (2017) 10:609. doi: 10.3389/fnins.2016.00609
79. Lin I-M, Fan S-Y, Yen C-F, Yeh Y-C, Tang T-C, Huang M-F, et al. Heart rate variability biofeedback increased autonomic activation and improved symptoms of depression and insomnia among patients with major depression disorder. *Clin Psychopharmacol Neurosci*. (2019) 17:222–32. doi: 10.9758/cpn.2019.17.2.222
80. Caldwell YT, Steffen PR. Adding HRV biofeedback to psychotherapy increases heart rate variability and improves the treatment of major depressive disorder. *Int J Psychophysiol*. (2018) 131:96–101. doi: 10.1016/j.ijpsycho.2018.01.001
81. Karavidas MK, Lehrer PM, Vaschillo E, Vaschillo B, Marin H, Buyske S, et al. Preliminary results of an open label study of heart rate variability biofeedback for the treatment of major depression. *Appl Psychophysiol Biofeedback*. (2007) 32:19–30. doi: 10.1007/s10484-006-9029-z
82. Siepmann M, Aykac V, Unterdörfer J, Petrowski K, Mueck-Weymann M. A pilot study on the effects of heart rate variability biofeedback in patients with depression and in healthy subjects. *Appl Psychophysiol Biofeedback*. (2008) 33:195–201. doi: 10.1007/s10484-008-9064-z

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Neural Correlates of Auditory Hypersensitivity in Fragile X Syndrome

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The mechanisms underlying the common association between autism spectrum disorders (ASD) and sensory processing disorders (SPD) are unclear, and treatment options to reduce atypical sensory processing are limited. Fragile X Syndrome (FXS) is a leading genetic cause of intellectual disability and ASD behaviors. As in most children with ASD, atypical sensory processing is a common symptom in FXS, frequently manifesting as sensory hypersensitivity. Auditory hypersensitivity is a highly debilitating condition in FXS that may lead to language delays, social anxiety and ritualized repetitive behaviors. Animal models of FXS, including *Fmr1* knock out (KO) mouse, also show auditory hypersensitivity, providing a translation relevant platform to study underlying pathophysiological mechanisms. The focus of this review is to summarize recent studies in the *Fmr1* KO mouse that identified neural correlates of auditory hypersensitivity. We review results of electroencephalography (EEG) recordings in the *Fmr1* KO mice and highlight EEG phenotypes that are remarkably similar to EEG findings in humans with FXS. The EEG phenotypes associated with the loss of FMRP include enhanced resting EEG gamma band power, reduced cross frequency coupling, reduced sound-evoked synchrony of neural responses at gamma band frequencies, increased event-related potential amplitudes, reduced habituation of neural responses and increased non-phase locked power. In addition, we highlight the postnatal period when the EEG phenotypes develop and show a strong association of the phenotypes with enhanced matrix-metalloproteinase-9 (MMP-9) activity, abnormal development of parvalbumin (PV)-expressing inhibitory interneurons and reduced formation of specialized extracellular matrix structures called perineuronal nets (PNNs). Finally, we discuss how dysfunctions of inhibitory PV interneurons may contribute to cortical hyperexcitability and EEG abnormalities observed in FXS. Taken together, the studies reviewed here indicate that EEG recordings can be utilized in both pre-clinical studies and clinical trials, while at the same time, used to identify cellular and circuit mechanisms of dysfunction in FXS. New therapeutic approaches that reduce MMP-9 activity and restore functions of PV interneurons may succeed in reducing FXS sensory symptoms. Future studies should examine long-lasting benefits of developmental vs. adult interventions on sensory phenotypes.

Keywords: Fragile X Syndrome, autism spectrum disorders, sensory processing disorders, auditory processing, sensory hypersensitivity, matrix metalloproteinase, GABA

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INTRODUCTION

There is a strong association between autism spectrum disorders (ASD) and sensory processing disorders (SPD). Indeed, the latest diagnostic criteria for ASD includes atypical sensory function as a core deficit. Research findings in both humans with ASD and animal models of ASD suggest that abnormal sensory processing in early development may lead to a broader array of symptoms including abnormal anxiety, social, and hyperactive behaviors (1–5). Despite the association between ASD behaviors and SPD, little is known about underlying cellular and circuit mechanisms that links autism to sensory issues. This review focuses on recent studies of the auditory system in Fragile X Syndrome (FXS), the most common genetic cause of ASD-associated behaviors and makes the case that studying basic sensory processing has multiple advantages in terms of identifying translation-relevant neural correlates, while at the same time gaining insight into the circuit mechanisms that lead to symptoms.

FRAGILE X SYNDROME

Fragile X syndrome is a genetic disorder that affects ∼1 in 4,000 males and 1 in 8,000 females (6, 7). FXS results from the loss of Fragile X Mental Retardation protein (FMRP), an mRNA binding protein that targets key synaptic pathways. FMRP is reduced or absent in humans with FXS due to an expansion and hyper-methylation of CGG trinucleotide repeats in the promoter region of the *FMR1* gene (8). Individuals with FXS experience a wide array of symptoms including intellectual impairment, language delays, seizures, repetitive behaviors, social anxiety, and hyperactivity. Consistently, abnormal sensory sensitivity (typically hypersensitivity) is seen in humans with FXS. Approximately 15–33% of individuals with FXS meet the diagnostic criteria for autism, with ∼5% of autism cases attributed to FXS (9–12). Many symptoms of FXS and ASD are similar, suggesting that studies of neural mechanisms in FXS may be broadly informative.

WHY STUDY THE AUDITORY SYSTEM IN FXS?

Both humans with FXS, and a commonly used animal model of the condition, the *Fmr1* knockout (KO) mouse, show auditory hypersensitivity. Neural circuits involved in auditory processing, particularly those in the early stages of processing, are likely to be more conserved across humans and rodents than circuits involved in social and cognitive symptoms. There are many similarities between humans and rodents in the basic organization of the auditory system from subcortical areas to the primary auditory cortex. There is also a rich history of studying auditory system development. Given that FXS is a neurodevelopmental disorder, existing knowledge on normal auditory circuit development provides a strong basis to study circuits that underlie hypersensitivity. The auditory system and auditory-related symptomatology offer a translation-relevant platform to identify clinically-relevant phenotypes and study circuit mechanisms of deficits in FXS. Indeed, as reviewed below, studies of auditory cortical processing in humans with FXS and mouse models have found remarkable similarities across species.

Abbreviations: ASD, Autism Spectrum Disorders; Fmr1, Fragile X Mental Retardation 1 gene; FMRP, Fragile X Mental Retardation Protein; FXS, Fragile X Syndrome; EEG, Electroencephalography; ERP, Event Related Potential; ITPC, Inter-Trial Phase Coherence; KO, Knock Out; MMP-9, Matrix Metalloproteinase-9; P, Post-natal day; PNN, Perineuronal Nets; PV, Parvalbumin; SPD, Sensory Processing Disorder; STG, Superior Temporal Gyrus; STP, Single Trial Power; WT, Wild Type.

EEG PHENOTYPES RELATED TO SENSORY PROCESSING IN HUMANS WITH FXS

Many of the early studies of auditory hypersensitivity in humans with FXS focused on auditory event-related potential (ERP) recordings. ERP studies consistently showed enhanced amplitude of various components (e.g., N1, P2). Enhanced synchrony of population responses to individual tones is likely responsible for enlarged N1 component of ERPs observed in humans with FXS (13–20), which may be generated by specific cell types in the auditory and frontal cortex (21, 22). A study using MEG also revealed enlargement of the N100m [the MEG equivalent of the N1 in EEG (14)]. In addition, the habituation of the N1 component to repeated tones is reduced in humans with FXS (17, 23); and the P2 amplitude of the ERP is enhanced in FXS (18). The similarity in observed MEG and EEG phenotypes adds further validity to the findings. The increase in N1 and P2 amplitude may be related to neuroanatomical abnormalities in the superior temporal gyrus (STG) where the auditory cortex is located (24), and to white matter enlargement in the temporal lobe (25). The enhanced N1 amplitude is also consistent with functional imaging studies that show that the STG displays higher levels of activation in individuals with FXS (26). Behavioral auditory hypersensitivity may therefore result from altered cortical responses to sounds (cortical hyperexcitability) in humans with FXS. Both enhanced population responses to sounds and reduced habituation of cortical neurons to repeating sounds may lead to auditory hyperexcitability (27).

Human EEG Spectral Component Analysis and Relationship to Clinical Measures

More recent EEG studies in humans have examined spectral components of baseline and sound-evoked responses to identify deficits in neural oscillations that are associated with sensory and cognitive symptoms in FXS. Wang et al. (28) found that FXS patients ($n = 21$, mean age = 26.4, range 10–55 yrs) exhibited greater gamma frequency band power (30–80 Hz) in the resting state EEG compared to age matched controls ($n = 21$). There was a reduction in alpha-gamma amplitude coupling across electrodes in FXS that suggests reduced top-down cortico-cortical control in FXS (29). The gamma power abnormality was correlated with social and sensory processing difficulties as measured with Social Communication Questionnaire and Adolescent/Adult Sensory Profile scores. These data are consistent with the reduced alpha-increased

gamma power trends observed across ASDs (30). Ethridge et al. (31) replicated the gamma band finding in humans with FXS (n = 17, mean age = 26.2, range = 11–55) and showed that abnormalities in gamma power were related to more severe behavioral and psychiatric features and reductions in neurocognitive functions. In addition, test-retest data shows reliability of measures in a third group of humans with FXS (n = 38, mean age = 25.5, range = 10–53) (18). Taken together, the resting EEG gamma power and ERP amplitude phenotypes have been replicated multiple times, with indications of scalability and retest reliability, which is critical for biomarker development. Importantly, these data demonstrate a close relationship between EEG measures and clinical manifestations.

Although elevated gamma power is found consistently, additional studies are needed to address its relevance. For example, Wilkinson and Nelson (32) found elevated aperiodic power in the beta-gamma range (25–50 Hz) in a younger cohort of boys with FXS age 2.5–7 (mean ∼4 yrs). However, they found no association between gamma power and sensory hypersensitivity or adaptive behaviors. Rather, they found an association between elevated gamma power and improved language ability in boys with FXS, suggesting that the gamma elevation may reflect compensatory mechanisms in FXS (33). Given the links between gamma oscillations and sensory-cognitive functions, and the emerging evidence that aperiodic gamma power may reflect cortical activation and excitatory/inhibitory balance in the cortex (34), a comprehensive quantification of oscillatory and aperiodic gamma power in the resting EEG needs to be obtained and correlated with clinical scores across development to properly identify biomarkers for clinical use. Abnormal periodic and aperiodic gamma power may serve as specific biomarkers for stratification of patients and outcome measures for clinical trials.

The gamma power related to local network excitation may reduce the ability of the neural population to synchronize periodic gamma band activity. Indeed, Ethridge et al. (31) found specific deficits in the gamma synchronization by testing the ability of the neural generators of the EEG signals to phase lock to dynamic auditory stimuli called "chirp." The chirp stimulus is a tone whose amplitude is modulated by a sinusoid of linearly increased or decreased frequency in the 1–100 Hz range. The ability of the auditory system to phase lock consistently across trials to the different frequencies (1–100 Hz range) in the chirp is quantified as the inter-trial phase coherence (ITPC). Humans with FXS including ages in 12–57 range (mean ∼26 yrs) show reduced ITPC in the 30–50 Hz gamma frequencies, but enhanced non-phase locked baseline broadband gamma power as the chirp trial was ongoing. These findings were replicated in another study of control and FXS human subjects (mean ∼25 yrs, range 10–53) at a different clinical site and using different EEG equipment (18) compared to the Ethridge et al. (31) paper.

The abnormal responses of auditory cortex to sound that are present from early development may affect communications and language skills (35, 36). Indeed, humans with FXS show delays and abnormalities in expressive language skills [Reynell Developmental Language Scales—Roberts et al. (37)]. Individuals with FXS experience difficulty articulating words, poor co-articulation, substitutions, and omissions of words, reduction in the number of intelligible syllables produced, difficulty sequencing sounds, and echolalia (38–42). Similar language delays seen in autism may be associated with basic auditory processing abnormalities in early sensory cortical regions (43). Schmitt et al. (44) used a "talk/listen" paradigm and EEG recordings to address possible underpinnings of the expressive language deficits in FXS. In this task, EEGs were recorded when the subject either uttered a phoneme or passively listened to the same phoneme. In a healthy individual a suppression of ERP component amplitudes is normally observed when subjects say the phoneme compared to when they listen to it (so called N1 suppression) with a negative signal in the EEGs just before the speech sound is produced (pre-speech negativity). These changes are attributed to an efference copy from the motor generators to the speech perception regions of the brain. In contrast, FXS subjects showed reduced pre-speech negativity and elevated gamma power in frontal loci that were related to speech intelligibility when frontal and temporal EEG recordings were compared between controls and humans with FXS (44). There was also reduced frontotemporal coherence in the theta-alpha frequency bands just prior to speech production, but no difference in N1 suppression was observed during the speech production. These EEG data suggest that abnormal signaling between frontal and temporal cortical regions (45) may underlie the expressive speech deficits in FXS. Elevated gamma power in the pre-speech time window indicates the gamma phenotype described above in sensory regions is also seen more broadly, can be task-related, and may relate to broader cognitive deficits in FXS.

EEG PHENOTYPES RELATED TO SENSORY PROCESSING IN ANIMAL MODELS OF FXS

Recent implementation of new EEG technology for pre-clinical studies in awake and freely moving mice demonstrated that similar EEG phenotypes are also observed in animal models of FXS, mainly *Fmr1* KO mice (**Table 1**) (51, 58). Lovelace et al. (55) compared EEG recordings between adult WT and *Fmr1* KO mice on FVB background and showed elevated baseline gamma power, reduced phase locking at gamma band frequencies with the chirp stimuli, enhanced non-phase gamma band power during the chirp trials and enhanced N1 ERP amplitude. Enhanced gamma power, enhanced ERP amplitude and reduced gamma synchronization to chirp are also seen in adult *Fmr1* KO mice on the C57BL6 background (47, 55). In addition, enhanced baseline gamma power and impaired sound-responses were observed in young P21–P28 *Fmr1* KO mice from both backgrounds (52, 59), suggesting early development of the abnormal EEG phenotypes. Interestingly, the *Fmr1* KO mice showed a larger increase in gamma band power during movement (46), suggesting the possibility that the motor modulation of auditory cortex may be abnormal in FXS. Although abnormal habituation to sound was not reported in awake and freely moving mice, earlier EEG

studies in anesthetized adult *Fmr1* KO mice on the FVB strain showed reduced habituation of N1 with repeated stimulation (56). This phenotype has not been tested in younger mice or the C57 strain. While these EEG data were obtained with epidural screw electrodes, for more immediate translation relevance, recent studies using a 30-channel skull surface multielectrode array (MEA) recording technique showed essentially the same EEG phenotypes in the *Fmr1* KO mice (49). The increased number of recording sites, along with broader spatial coverage will now facilitate advanced EEG analysis, including cross-frequency and cross-region analysis in awake and freely moving mice to more closely relate to high-density human EEG studies.

Similar EEG phenotypes were also observed in the *Fmr1* KO rat model of FXS, which displayed enhanced baseline gamma band power, reduced alpha power and behavioral hyperactivity (57). In addition, sound-evoked response, more specifically ITPC when tested with click trains to elicit an auditory steady state response, also showed a decrease in the gamma oscillations in the *Fmr1* KO rat. The findings were consistent with reduced ITPC auditory steady state response observed in the *Fmr1* KO mouse in response to a 40 Hz click train (47). Interestingly, studies in juvenile *Fmr1* KO rat visual cortex showed that the typical switch from higher to lower frequency dominance in cortical response was impaired when the animal went from an active to a resting state (53). The high-frequency power remained elevated in the *Fmr1* KO rat compared to the WT counterparts yet again suggesting abnormal modulation of sensory cortex responses by movement states. The species similarity (humans, mice, and rats) in the EEG phenotypes and the specific frequency bands affected is remarkable, and could prove critically useful in developing similar outcome measures between pre-clinical and clinical trials, while at the same time facilitate discovery of underlying cellular and circuit mechanisms, and new therapeutic interventions in the animal models. Future studies need to validate selected EEG phenotypes as biomarkers by performing studies on robustness, scalability, tolerance to settings and equipment and sensitivity to drug treatments.

SYSTEMS, CIRCUIT, AND CELLULAR MECHANISMS OF AUDITORY HYPERSENSITIVITY IN FXS

Considering clinical relevance of the sensory hypersensitivity, several recent studies are focused on deciphering cellular and circuit mechanisms underlying it, utilizing both *in vivo* and *in vitro* approaches. Rotschafer and Razak (60) showed that individual neurons in the auditory cortex of *Fmr1* KO mice responded with more action potentials to tones than in WT mice, using *in vivo* single unit recordings. Although the onset responses were similar across the genotypes, the responses were prolonged and continued well after sound offset in *Fmr1* KO neurons, but not in WT neurons. This indicates an increased duration of responses in the *Fmr1* KO mouse cortex, and may be related to the observed increase in baseline corrected single trial power (18, 46) and increase in resting gamma power in EEG responses (46, 50). Rotschafer and Razak (60) also showed that the frequency tuning receptive field of cortical neurons was broader in the *Fmr1* KO mice. This indicates that for the same tone, more neurons will be synchronously activated in the auditory cortex of *Fmr1* KO mice compared to WT mice and may underlie enhanced N1 amplitudes of ERPs, and the larger STG activation in humans with FXS (26). These increases in neural responses may arise from abnormal activation of inhibitory neurons (61, 62). In these studies, an examination of excitatory (E) and inhibitory (I) inputs to neurons in the somatosensory cortex provides important clues in terms of underlying circuit mechanisms of cortical neuron hyper-responsiveness. The strength of cortical E $\rightarrow$ E and I $\rightarrow$ E synaptic connections is shown to be relatively normal in the developing somatosensory cortex of *Fmr1* KO mice. However, cortical E $\rightarrow$ I synaptic communication is reduced leading to reduced activation of inhibitory neurons, that may lead to increased excitation in the network. Local hyperconnectivity between pyramidal neurons due to deficient pruning may also lead to increased synchrony and responses in the network (63).

Development of Electrophysiological Abnormalities in *Fmr1* KO Mice

To investigate developmental trajectory of the abnormal phenotypes, Wen et al. (59) compared neuronal responses to sound between *Fmr1* KO and WT mice and identified the postnatal (P14–P21) window during which cortical responses began to diverge in the auditory cortex of *Fmr1* KO mice. Single unit recordings showed that responses were similar in cortical neurons of WT and *Fmr1* KO mice at P14. However, the responses were larger in the *Fmr1* KO cortex at P21. This indicates that just after hearing onset ($\sim$P10) in mice, the abnormal development of circuits induced by auditory experience may underlie cortical hypersensitivity in the *Fmr1* KO mice. The *Fmr1* KO rat visual cortex, as well, shows a divergence of responses around the period of eye-opening (53). The P14–21 developmental window coincides with the age during which the excitatory and inhibitory connections mature in the mouse auditory cortex acquiring adult-like characteristics (64, 65). Perturbation of auditory experience during this window using tone exposure leads to tonotopic plasticity in the WT mouse, but such critical period plasticity is disrupted in *Fmr1* KO mice (66), possibly due to impaired stability of long-term potentiation (67).

Disentangling Cortical vs. Subcortical Contributions to Auditory Hypersensitivity

Besides auditory cortex, FMRP expression is detected across the entire auditory neuraxis, with the possible exception of the cochlea (68–70). While the preponderance of studies in both humans and animal models have focused on the cortex, both subcortical site abnormalities and/or local cortical processing abnormalities may contribute to the phenotypes recorded in the cortex (70–73). Indeed, both the brainstem and midbrain auditory nuclei show abnormal synaptic markers and electrophysiological responses. The inferior colliculus shows broader frequency tuning curves, and enhanced responses to tones and amplitude modulated sounds (73). As in the cortex, these abnormalities develop between P14 and P21, a time window

TABLE 1 | Species similarity in EEG phenotypes.

EEG phenotype	*Fmr1* KO mouse/rat	Humans with FXS
Resting (baseline) EEG gamma band power	Increased/increased (46–54)	Increased (18, 28, 30, 31)
Non-phase-locked power in the gamma band	Increased (47, 48, 50, 52)	Increased (18, 30, 31)
ERP N1 amplitude	Increased (46, 49, 50, 55, 56)	Increased (13, 15, 16, 19)
ERP N1 habituation	Decreased (56)	Decreased (18, 23)
Phase locking to chirp stimuli in the gamma band (ITPC)	Decreased (46–49, 54)	Decreased (18, 31)
Phase locking in 40 Hz auditory steady state response (ITPC)	Decreased/decreased (48, 57)	*Non tested*
Cross-frequency coupling	Reduced alpha-gamma coupling (52)	Reduced alpha-gamma coupling (28)

The table lists the major EEG findings in rodents and humans that could be used as EEG correlates of sensory hypersensitivity. The direction of change is remarkably similar across species. ITPC, Inter-Trial Phase Coherence.

during which intracollicular intrinsic inhibition matures to adult-like levels (74). More neurons exhibit cFos immunoreactivity in response to sounds in the inferior colliculus, indicative of enhanced cell activation, suggesting that population synchrony may be elevated in this region. The hyperexcitability of the inferior colliculus during early development is consistent with the suggestion that this midbrain region is involved in the generation of audiogenic seizures, a commonly studied phenotype in *Fmr1* KO mice (75). Supporting the role of midbrain in increased susceptibility to the audiogenic seizures, re-expression of FMRP in the glutamatergic neurons of inferior colliculus, in the Fmr1 KO mouse, prevents audiogenic seizures. Conversely, the deletion of Fmr1 in glutamatergic neurons of the inferior colliculus triggers audiogenic seizures. These data suggest that subcortical auditory sites show hyperexcitability, at least during early development.

While the brainstem and midbrain studies suggest that cortical hyperexcitability may reflect subcortical abnormalities, *in vitro* slice studies also indicate that local cortical processing may be abnormal. Goswami et al. (76) found that layer 2/3 circuits were hyperexcitable and showed increased gamma power in layers 2/3 and 5 in auditory cortical slices from *Fmr1* KO mice following optogenetic activation of local circuits. These studies were consistent with *in vivo* studies of resting and sound driven activity and showed increased synchrony between layers 2/3 and 5. Considering that subcortical inputs are absent in slice electrophysiological studies, these data indicate local cortical deficits or reflect compensatory plasticity of intrinsic properties during the development of the mice from which slices were taken. To investigate the contribution of local cortical deficits *in vivo*, Lovelace et al. (47) examined the effects of *Fmr1* deletion only from excitatory neurons in the forebrain using the *Nex1* promoter. In this mouse model of FXS, FMRP expression was normal in the midbrain and thalamus, while cortical excitatory neurons showed loss of FMRP allowing for an examination of local cortical abnormalities following FMRP loss. EEG resting gamma power, and non-phase locked power in sound-evoked trials were elevated, as seen in global *Fmr1* KO mice. However, the chirp-induced gamma synchronization (ITPC) was normal. These data indicate that a mixture of local cortical processing deficits and inherited deficits from subcortical sites lead to the observed cortical phenotypes, pointing to the need for a balanced investigation across the auditory neuraxis. Indeed, very little is known about subcortical auditory responses in humans with FXS. Interestingly, hyperactive locomotor behavior, but no changes in anxiety-like behaviors, was observed in mice with forebrain excitatory-specific *Fmr1* deletion, pointing to combined cortical and subcortical contributions to behavioral deficits in FXS.

Cellular Mechanisms of Auditory Hypersensitivity in *Fmr1* KO Mice—The MMP-9 Link

Delving more into the cellular mechanisms of abnormal cortical responses, several studies reported abnormal development and function of specific GABAergic neuron subtype parvalbumin (PV)-expressing interneurons. In particular, PV inhibitory interneurons in the cortex have been implicated in sensory hypersensitivity and abnormal sensory processing in *Fmr1* KO mice in both visual and somatosensory systems (77–79). Gibson et al. (61) found a significant reduction in local excitatory drive on fast-spiking interneurons (putative PV neurons) in layer 4 of the somatosensory cortex. PV-expressing interneurons provide synchronous inhibition of multiple neighboring pyramidal cells, a process that is thought to be important in the generation of the narrowband gamma frequency rhythm (80–82). These cells may also be involved in desynchronizing higher frequency broadband gamma activity, implicating PV cells in the observed EEG phenotypes in FXS (83, 84). A characteristic structural feature of PV cells in the cortex is the preponderance of a specialized extracellular matrix structures called the perineuronal nets (PNNs) (59). PNNs are thought to increase excitability of PV cells (85) and thereby increase network inhibition. PNNs formation around PV cells also coincides with the closure of critical period plasticity windows in sensory cortices (86–89).

Auditory cortical hyperexcitability in FXS may arise from abnormal development of PV cells and PNNs during the P14–P21 window, the time window of divergence in cortical responses in *Fmr1* KO mice (59). A reduced density of PV-expressing cells and the numbers of PNN-enwrapped PV cells in the *Fmr1* KO mouse cortex at P21 may affect PV cell function and cortical inhibition. PNNs are dynamic structures and can be degraded by the activity of multiple proteases, including matrix metalloproteinase-9 (MMP-9). MMP-9 is a zinc-dependent endopeptidase that is

found in many cell types, including neurons and glia (90). Among a large family of MMPs, MMP-9, MMP-2, and MMP-3 are widely expressed in the CNS and the expression of MMP-9 is regulated during development (90).

MMP-9 is a translational target of FMRP (91) and in the absence of FMRP, there is increased activity of MMP-9 across multiple brain regions and developmental periods in *Fmr1* KO mice (59, 92, 93). Increased MMP-9 levels and activity were also observed in FXS human samples (92, 94). In addition, neural circuit deficits in *Drosophila* model of FXS were linked to MMPs and removal of *mmp1,* that encodes a secreted form of mmp in drosophila, ameliorated synaptic architecture defects at the neuromuscular junctions of *dfmr1 null* mutants (95). While reduction or loss of MMP-9 expression in *Fmr1* KO mice reduced FXS-like symptoms (59, 92), MMP-9 overexpression in mice resulted in FXS-like symptoms (94). To test the role of MMP-9 in abnormal PV and PNN development, Wen et al. (59) utilized a genetic approach allowing to reduce MMP-9 to the normal levels in the *Fmr1* KO mice. In these mice, not only PNNs were restored to normal levels, in particular around PV-expressing cells, cortical tone-driven responses were also normalized. In addition, abnormal sensory gating as tested with the pre-pulse inhibition of acoustic startle was also improved in these mice (93). Interestingly, even a complete removal of MMP-9 in the *Fmr1* KO mice improved ERP habituation (56). The effectiveness of minocycline treatment in normalizing abnormal ERP habituation in FXS humans was also linked to the reduction of MMP-9 activity (17, 96), suggesting that elevated levels of MMP-9 may contribute to auditory hyperexcitability in FXS. Increased cortical MMP-9 activity and abnormal PV/PNN development were also observed in forebrain excitatory neuron-specific *Fmr1* KO mice (47), suggesting a key role of cortical excitatory neurons in the dysfunction of PV interneurons, enhanced MMP-9 activity and abnormal PNN development. The loss of FMRP in excitatory neurons lead to reduced excitatory innervation of PV cells (61) and PNN loss *via* enhanced MMP-9 activity (47), both of which can affect PV cell functions and cortical inhibition resulting in EEG gamma band abnormalities. Consistent with the role of PV hypofunction in cortical hyperexcitability, enhancing PV cell function in the visual cortex of *Fmr1* KO mice corrected orientation tuning of excitatory neurons and improved mouse performance in a visual perceptual learning task (78).

Therapeutics to Reduce Sensory Hypersensitivity

Given the strong evidence linking dysregulation of MMP-9 activity to the development of auditory cortex hyperexcitability, this pathway may serve as a potential therapeutic target to reduce sensory hypersensitivity. Minocycline is an FDA-approved antibiotic and a known inhibitor of MMP-9. Minocycline treatment in humans with FXS improved ERP habituation responses (17), and open label studies have shown significant functional improvements in FXS (97). A randomized placebo-controlled study of minocycline showed improvement in Clinical Global Impression Scale compared to placebo and greater improvement in anxiety and mood-related behaviors on the Visual Analog Scale (98).

Several studies have also shown benefits of minocycline treatment in the mouse and the drosophila models of FXS (95). For example, both minocycline treatment and genetic reduction of MMP-9 normalized the rate of ultrasonic vocalizations in *Fmr1* KO mice when paired with a receptive female (99, 100). Minocycline reduced audiogenic seizures, hyperactivity and anxiety-like behaviors in both young and adult *Fmr1* KO mice, but the effects lasted longer when the treatment was given at a young age (101). In contrast, adult mice had to be treated continuously for sustained benefits. These data point to an important element of treatment design –age of administration.

In addition to the improvements in mouse behaviors, a 10-day treatment of adult *Fmr1* KO mice with minocycline also influenced EEG phenotypes (47). By testing resting EEG, ERPs, auditory steady state and chirp response ITPC and non-phase locked power, this study found beneficial effects of minocycline over vehicle treatment in all phenotypes, except resting gamma EEG power. Minocycline treatment increased gamma synchronization in response to auditory stimuli, and reduced sound-evoked power of auditory ERPs in *Fmr1* KO mice compared to vehicle treatment. Although resting gamma power was reduced by minocycline, it was also reduced by vehicle treatment. Because minocycline has multiple targets besides MMP-9, including apoptotic pathway and microglia, it is necessary to test more specific inhibitors. Toward that goal, Pirbhoy et al. (52) tested acute treatment with SB-3CT, a MMP2/9 inhibitor, and demonstrated improved ITPC to auditory stimuli, enhanced PNN formation, and increased PV levels and TrkB phosphorylation in the auditory cortex of *Fmr1* KO mice. Importantly, the reduction of MMP2/9 activity also improved mouse behavior as tested in the open field and elevated plus maze. Good sensitivity and reproducibility of EEG recordings provide a scientific justification for future use of EEG outcome measures in pre-clinical studies, including translationally relevant MEA EEG recordings. Jonak et al. (54) showed that an orally active phosphodiesterase 10A (PDE10A) inhibitor (14-day treatment) normalized the chirp ITPC in *Fmr1* KO mice even at a low dose (0.5 mg/kg) without causing any sedation or effects on baseline EEG power. Taken together, these data indicate that sound-evoked EEG responses may be more sensitive measures, compared to resting EEG measures, to isolate drug effects from placebo in humans with FXS. Minocycline or other MMP-9 inhibitors show much promise in reducing sensory issues in FXS and selecting sensitive outcome measures based on the mouse EEG data may prove useful in designing statistically powerful clinical trials.

Kulinich et al. (55) also explored a non-pharmacological approach in reducing sensory hypersensitivity, in particular, therapeutic effects of reduced sound exposure during the P14–P21 developmental period, when auditory cortical hyperexcitability was first observed. Surprisingly, development of *Fmr1* KO mice in a sound-attenuated environment did not reduce abnormal phenotypes, and in some cases exacerbated the symptoms (55). However, cortical correlates of auditory hypersensitivity were reduced when the mice were exposed

to repeated tones at a rate of 5 Hz during this developmental window. Development of PV cells and PNNs, dendritic spines, TrkB phosphorylation and ERP amplitudes were normalized following the developmental sound exposure. These data suggest that developmental sound exposure during the critical period window, and not sound attenuation, may serve as a potential treatment option either alone, or in combination with pharmacological approaches.

Summary—Quadruple Hit Model of Auditory Hypersensitivity in FXS

Auditory hypersensitivity is a highly debilitating and commonly associated condition in humans with FXS (102). The *Fmr1* KO mouse model of FXS also shows this behavioral phenotype providing a strong basis for examining mechanisms that may help to develop new therapeutic approaches in humans. At a functional level, the remarkable similarities in EEG phenotypes are evident across humans and rodents, including increased gamma band resting power, reduced phase locking to time varying and steady state auditory stimuli but increased non-phase locked power, increased ERP amplitude and reduced habituation of ERPs to repeated stimuli (**Table 1**). The specificity, reproducibility and sensitivity of these EEG measures provide a strong rationale for using EEG outcomes in pre-clinical trials in mice. Importantly, the scalability and clinical correlations in human EEG work supports widespread use of similar EEG outcomes in clinical studies to see real-world benefits in humans with FXS.

Based on studies of the circuit mechanisms underlying auditory hypersensitivity in FXS, we emphasize a "quadruple hit" model to explain auditory hypersensitivity: (1) individual cortical neurons are hyper-responsive to sounds (59, 60); (2) more cortical neurons respond synchronously to the same sound (60); (3) habituation of cortical neurons to repeated/continuous sounds is reduced (56); (4) background cortical activity is increased (46, 49, 50). These four phenotypes create a milieu of background noise, particularly manifesting as elevated broadband gamma noise, above which cortical neurons need to increase their responses to improve signal to noise ratio in information transfer. From a cellular and molecular perspective, recent studies from our and other groups implicated MMP-9 and PV-expressing inhibitory interneurons in abnormal circuit functions that underlie cortical hyperexcitability (47, 52, 59) as follows:

Loss of FMRP → Increased MMP-9 → Reduced PNNs around PV cells → Reduced excitability of PV cells → Reduced inhibition of cortical networks → Abnormal gamma synchrony and cortical hyperexcitability.

Future Studies

1. The functional deficits in sensory processing may emerge during specific developmental windows due to abnormal changes in circuit development providing an opportunity to target specific circuits for treatments during these windows (36). Given the vast literature on the critical role of developmental sensory experience that shapes brain structure and function over the lifespan, it is highly likely that early developmental treatments to normalize sensory circuit development will be most effective. However, it remains to be tested whether early developmental therapeutic interventions can normalize sensory processing with long-lasting benefits. It is also unclear whether early postnatal interventions to normalize sensory processing will have broader impacts and prevent abnormal behaviors in humans with FXS, such as anxiety, impaired social communication, delayed language function, and hyperactivity. Early reversal of sensory processing deficits may result in broad-acting benefits, an idea that remains untested in FXS.
2. There is a significant number of molecular targets considered as a treatment for FXS (103, 104). However, clinical trials have either failed or are inconclusive (105), contributing to mounting frustration in the FXS community. While very recent studies using phosphodiesterase inhibitors are promising (54, 106), continued efforts to understand how multiple pathways implicated in FXS interact, leading to circuit dysfunction and abnormal behaviors. One earlier theory suggests enhanced mGluR5-dependent protein synthesis in the *Fmr1* KO mouse model providing a possible link between over-activated mGluR5 and enhanced protein translation in neurons lacking FMRP (107). A recent study also showed a new link between mGluR5 and MMP-9 reporting that deleting or blocking mGluR5 can decrease MMP-9 activity resulting in an elevated (almost doubling) number of PNNs in the somatosensory cortex (108). These data suggest that the increase in mGluR5 activity can lead to increased MMP-9 activity and PNN loss in FXS, suggesting a potential link between the mGluR5 and MMP-9 theories of FXS hyperexcitability. Future studies should explore these links in greater detail to determine whether MMP-9 acts downstream of mGluR5 and can be targeted therapeutically alongside or instead of mGluR5 antagonists, helping to reduce any buildup of tolerance and side-effects.
3. There is a predominant focus on the neocortex and hippocampus in studies of FXS and ASD, which is particularly true in humans. However, our investigations of the mechanisms of auditory dysfunction in FXS indicate that the cortically recorded phenotypes may reflect a mixture of local circuit deficits and subcortical deficits. A systematic investigation of deficits in subcortical processing and their developmental time course using transgenic mouse lines and promoters that allow spatial and cell-type-specific deletion or re-expression of FMRP could facilitate these studies in animal models. In humans, frequency following responses (FFR) which likely originate in the midbrain/brainstem region (109, 110) can be recorded to identify differential subcortical processing in FXS and ASD.
4. The ability of early sound exposure, but not sound attenuation, to reduce cortical hyperexcitability symptoms suggests that developmental trajectories of atypical sensory processing need to be investigated across closely spaced developmental ages. Examination of deficits at a single age or a small number of ages may miss the main cause of pathology early on, and only record manifestation of compensatory

mechanisms (32, 33), which may be indirectly altered by the genetic mutation and can be beneficial (111–113).

5. The excitement around developments in the field of gene therapy indicates this approach may allow re-expression of FMRP in the near future (114, 115). However, our understanding of the function of FMRP at different ages remains underwhelming. In particular, it is unclear whether adults may benefit from FMRP re-expression, or if re-expression has to occur during embryonic or early postnatal development. There is no study comparing the developmental vs. adult effects of FMRP expression in the same model, using the same outcome measures. One published paper on this topic showed that acute expression of FMRP in adult prefrontal cortex is sufficient to elicit normal learning of adult *Fmr1* KO mice in a prefrontal cortex dependent task (116). Despite the strong evidence for early developmental abnormalities in FXS, whether targeted interventions at this age provide long-lasting benefits is also unclear. In Angelman Syndrome, reactivation of Ube3A at different developmental time points has a phenotype-specific effect, but in Rett Syndrome benefits are seen for both early and late corrections of the deficits (117–119). These data from other forms of ASD indicate that a systematic study of effects of FMRP re-expression at different ages, and using a broad range of structural, functional and behavioral outcome measures is necessary. The findings reviewed here indicate that studies of sensory hypersensitivity may provide a tangible and translationally relevant niche to address these urgent issues.

AUTHOR CONTRIBUTIONS

KR, DB, and IE contributed to the writing of the review. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Green SA, Ben-Sasson A. Anxiety disorders and sensory over-responsivity in children with autism spectrum disorders: is there a causal relationship?. *J Autism Dev Disord.* (2010) 40:1495–504. doi: 10.1007/s10803-010-1007-x
2. Uljarević M, Lane A, Kelly A, Leekam S. Sensory subtypes and anxiety in older children and adolescents with autism spectrum disorder. *Autism Res.* (2016) 9:1073–8. doi: 10.1002/aur.1602
3. Rais M, Binder DK, Razak KA, Ethell IM. Sensory processing phenotypes in fragile X syndrome. *ASN Neuro.* (2018) 10:1759091418801092. doi: 10.1177/1759091418801092
4. Orefice LL, Zimmerman AL, Chirila AM, Sleboda SJ, Head JP, Ginty DD. Peripheral mechanosensory neuron dysfunction underlies tactile and behavioral deficits in mouse models of ASDs. *Cell.* (2016) 166:299–313. doi: 10.1016/j.cell.2016.05.033
5. Orefice LL, Mosko JR, Morency DT, Wells MF, Tasnim A, Mozeika SM, et al. Targeting peripheral somatosensory neurons to improve tactile-related phenotypes in ASD models. *Cell.* (2019) 178:867–86. doi: 10.1016/j.cell.2019.07.024
6. Hagerman RJ, Berry-Kravis E, Kaufmann WE, Ono MY, Tartaglia N, Lachiewicz A, et al. Advances in the treatment of fragile X syndrome. *Pediatrics.* (2009) 123:378–90. doi: 10.1542/peds.2008-0317
7. Hagerman RJ, Berry-Kravis E, Hazlett HC, Bailey DB, Moine H, Kooy RF, et al. Fragile X syndrome. *Nat Rev Dis Primers.* (2017) 3:1–19. doi: 10.1038/nrdp.2017.65
8. O'Donnell WT, Warren ST. A decade of molecular studies of fragile X syndrome. *Annu Rev Neurosci.* (2002) 25:315–38. doi: 10.1146/annurev.neuro.25.112701.142909
9. Bailey DB, Mesibov GB, Hatton DD, Clark RD, Roberts JE, Mayhew L. Autistic behavior in young boys with fragile X syndrome. *J Autism Dev Disord.* (1998) 28:499–508. doi: 10.1023/A:1026048027397
10. Cohen D, Pichard N, Tordjman S, Baumann C, Burglen L, Excoffier E, et al. Specific genetic disorders and autism: clinical contribution towards their identification. *J Autism Dev Disord.* (2005) 35:103–16. doi: 10.1007/s10803-004-1038-2
11. Niu M, Han Y, Dy ABC, Du J, Jin H, Qin J, et al. Autism symptoms in fragile X syndrome. *J Child Neurol.* (2017) 32:903–9. doi: 10.1177/0883073817712875
12. Abbeduto L, Thurman AJ, McDuffie A, Klusek J, Feigles RT, Brown WT, et al. ASD comorbidity in fragile X syndrome: symptom profile and predictors of symptom severity in adolescent and young adult males. *J Autism Dev Disord.* (2019) 49:960–77. doi: 10.1007/s10803-018-3796-2
13. St. Clair DMS, Blackwood DH, Oliver CJ, Dickens P. P3 abnormality in fragile X syndrome. *Biol Psychiatry.* (1987) 22:303–12. doi: 10.1016/0006-3223(87)90148-X
14. Rojas DC, Benkers TL, Rogers SJ, Teale PD, Reite ML, Hagerman RJ. Auditory evoked magnetic fields in adults with fragile X syndrome. *Neuroreport.* (2001) 12:2573–6. doi: 10.1097/00001756-200108080-00056
15. Castrén M, Pääkkönen A, Tarkka IM, Ryynänen M, Partanen J. Augmentation of auditory N1 in children with fragile X syndrome. *Brain Topogr.* (2003) 15:165–71. doi: 10.1023/A:1022606200636
16. Van der Molen MJW, Van der Molen MW, Ridderinkhof KR, Hamel BCJ, Curfs LMG, Ramakers GJA. Auditory change detection in fragile X syndrome males: a brain potential study. *Clin Neurophysiol.* (2012) 123:1309–18. doi: 10.1016/j.clinph.2011.11.039
17. Schneider A, Leigh MJ, Adams P, Nanakul R, Chechi T, Olichney J, et al. Electrocortical changes associated with minocycline treatment in fragile X syndrome. *J Psychopharmacol.* (2013) 27:956–63. doi: 10.1177/0269881113494105
18. Ethridge LE, De Stefano LA, Schmitt LM, Woodruff NE, Brown KL, Tran M, et al. Auditory EEG biomarkers in fragile X syndrome: clinical relevance. *Front Integr Neurosci.* (2019) 13:60. doi: 10.3389/fnint.2019.00060
19. Knoth IS, Lippé S. Event-related potential alterations in fragile X syndrome. *Front Human Neurosci.* (2012) 6:264. doi: 10.3389/fnhum.2012.00264
20. Ethridge L, Thaliath A, Kraff J, Nijhawan K, Berry-Kravis E. Development of neural response to novel sounds in fragile x syndrome: potential biomarkers. *Am J Intellectual Dev Disabil.* (2020) 125:449–64. doi: 10.1352/1944-7558-125.6.449

21. Hari R, Kaila K, Katila T, Tuomisto T, Varpula T. Interstimulus interval dependence of the auditory vertex response and its magnetic counterpart: implications for their neural generation. *Electroencephal Clin Neurophysiol.* (1982) 54:561–9. doi: 10.1016/0013-4694(82)90041-4
22. Näätänen R, Picton T. The N1 wave of the human electric and magnetic response to sound: a review and an analysis of the component structure. *Psychophysiology.* (1987) 24:375–425. doi: 10.1111/j.1469-8986.1987.tb00311.x
23. Ethridge LE, White SP, Mosconi MW, Wang J, Byerly MJ, Sweeney JA. Reduced habituation of auditory evoked potentials indicate cortical hyper-excitability in Fragile X Syndrome. *Transl Psychiatry.* (2016) 6:e787. doi: 10.1038/tp.2016.48
24. Reiss AL, Lee J, Freund L. Neuroanatomy of fragile X syndrome: the temporal lobe. *Neurology.* (1994) 44:1317–7. doi: 10.1212/WNL.44.7.1317
25. Hazlett HC, Poe MD, Lightbody AA, Styner M, MacFall JR, Reiss AL, et al. Trajectories of early brain volume development in fragile X syndrome and autism. *J Am Acad Child Adolescent Psychiatry.* (2012) 51:921–33. doi: 10.1016/j.jaac.2012.07.003
26. Hall SS, Walter E, Sherman E, Hoeft F, Reiss AL. The neural basis of auditory temporal discrimination in girls with fragile X syndrome. *J Neurodev Disord.* (2009) 1:91–9. doi: 10.1007/s11689-009-9007-x
27. Schmid S, Wilson DA, Rankin CH. Habituation mechanisms and their importance for cognitive function. *Front Integr Neurosci.* (2015) 8:97. doi: 10.3389/fnint.2014.00097
28. Wang J, Ethridge LE, Mosconi MW, White SP, Binder DK, Pedapati EV, et al. A resting EEG study of neocortical hyperexcitability and altered functional connectivity in fragile X syndrome. *J Neurodev Disord.* (2017) 9:1–12. doi: 10.1186/s11689-017-9191-z
29. Von Stein A, Sarnthein J. Different frequencies for different scales of cortical integration: from local gamma to long range alpha/theta synchronization. *Int J Psychophysiol.* (2000) 38:301–13. doi: 10.1016/S0167-8760(00)00172-0
30. Wang J, Barstein J, Ethridge LE, Mosconi MW, Takarae Y, Sweeney JA. Resting state EEG abnormalities in autism spectrum disorders. *J Neurodev Disord.* (2013) 5:1–14. doi: 10.1186/1866-1955-5-24
31. Ethridge LE, White SP, Mosconi MW, Wang J, Pedapati EV, Erickson CA, et al. Neural synchronization deficits linked to cortical hyper-excitability and auditory hypersensitivity in fragile X syndrome. *Mol Autism.* (2017) 8:1–11. doi: 10.1186/s13229-017-0140-1
32. Wilkinson CL, Nelson CA. Increased aperiodic gamma power in young boys with Fragile X Syndrome is associated with better language ability. *Mol Autism.* (2021) 12:1–15. doi: 10.1186/s13229-021-00425-x
33. Antoine MW, Langberg T, Schnepel P, Feldman DE. Increased excitation-inhibition ratio stabilizes synapse and circuit excitability in four autism mouse models. *Neuron.* (2019) 101:648–61. doi: 10.1016/j.neuron.2018.12.026
34. Donoghue T, Haller M, Peterson EJ, Varma P, Sebastian P, Gao R, et al. Parameterizing neural power spectra into periodic and aperiodic components. *Nat Neurosci.* (2020) 23:1655–65. doi: 10.1038/s41593-020-00744-x
35. Abbeduto L, Brady N, Kover ST. Language development and fragile X syndrome: profiles, syndrome-specificity, and within-syndrome differences. *Mental Retardation Dev Disabil Res Rev.* (2007) 13:36–46. doi: 10.1002/mrdd.20142
36. Razak KA, Dominick KC, Erickson CA. Developmental studies in fragile X syndrome. *J Neurodev Disord.* (2020) 12:1–15. doi: 10.1186/s11689-020-09310-9
37. Roberts JE, Mirrett P, Burchinal M. Receptive and expressive communication development of young males with fragile X syndrome. *Am J Mental Retardation.* (2001) 106:216–30. doi: 10.1352/0895-8017(2001)106<0216:RAECDO>2.0.CO;2
38. Largo RH, Schinzel A. Developmental and behavioural disturbances in 13 boys with fragile X syndrome. *Euro J Pediatrics.* (1985) 143:269–75. doi: 10.1007/BF00442299
39. Hanson DM, Jackson, III AW, Hagerman RJ, Opitz JM, Reynolds JF. Speech disturbances (cluttering) in mildly impaired males with the Martin-Bell/fragile X syndrome. *Am J Med Genet.* (1986) 23:195–206. doi: 10.1002/ajmg.1320230114
40. Belser RC, Sudhalter V. Conversational characteristics of children with fragile X syndrome: repetitive speech. *Am J Mental Retardation.* (2001) 106:28–38. doi: 10.1352/0895-8017(2001)106<0028:CCOCWF>2.0.CO;2
41. Roberts J, Price J, Barnes E, Nelson L, Burchinal M, Hennon EA, et al. Receptive vocabulary, expressive vocabulary, and speech production of boys with fragile X syndrome in comparison to boys with Down syndrome. *Am J Mental Retardation.* (2007) 112:177–93. doi: 10.1352/0895-8017(2007)112177:RVEVAS2.0.CO;2
42. Barnes E, Roberts J, Long SH, Martin GE, Berni MC, Mandulak KC, et al. Phonological accuracy and intelligibility in connected speech of boys with fragile X syndrome or Down syndrome. *J Speech Lang Hear Res.* (2009) 52:1048–61. doi: 10.1044/1092-4388(2009/08-0001)
43. Del Rincon PN. Autism: alterations in auditory perception. *Rev Neurosci.* (2008) 19:61–78. doi: 10.1515/REVNEURO.2008.19.1.61
44. Schmitt LM, Wang J, Pedapati EV, Thurman AJ, Abbeduto L, Erickson CA, et al. A neurophysiological model of speech production deficits in fragile X syndrome. *Brain Commun.* (2020) 2:fcz042. doi: 10.1093/braincomms/fcz042
45. Schneider DM, Nelson A, Mooney R. A synaptic and circuit basis for corollary discharge in the auditory cortex. *Nature.* (2014) 513:189–94. doi: 10.1038/nature13724
46. Lovelace JW, Ethell IM, Binder DK, Razak KA. Translation-relevant EEG phenotypes in a mouse model of Fragile X Syndrome. *Neurobiol Dis.* (2018) 115:39–48. doi: 10.1016/j.nbd.2018.03.012
47. Lovelace JW, Rais M, Palacios AR, Shuai XS, Bishay S, Popa O, et al. Deletion of Fmr1 from forebrain excitatory neurons triggers abnormal cellular, EEG, and behavioral phenotypes in the auditory cortex of a mouse model of fragile X syndrome. *Cereb Cortex.* (2020) 30:969–88. doi: 10.1093/cercor/bhz141
48. Lovelace JW, Ethell IM, Binder DK, Razak KA. Minocycline treatment reverses sound evoked EEG abnormalities in a mouse model of Fragile X Syndrome. *Front Neurosci.* (2020) 14:771. doi: 10.3389/fnins.2020.00771
49. Jonak CR, Lovelace JW, Ethell IM, Razak KA, Binder DK. Multielectrode array analysis of EEG biomarkers in a mouse model of Fragile X Syndrome. *Neurobiol Dis.* (2020) 138:104794. doi: 10.1016/j.nbd.2020.104794
50. Wen TH, Lovelace JW, Ethell IM, Binder DK, Razak KA. Developmental changes in EEG phenotypes in a mouse model of fragile X syndrome. *Neuroscience.* (2019) 398:126–43. doi: 10.1016/j.neuroscience.2018.11.047
51. Sinclair D, Featherstone R, Naschek M, Nam J, Du A, Wright S, et al. GABA-B agonist baclofen normalizes auditory-evoked neural oscillations and behavioral deficits in the Fmr1 knockout mouse model of fragile X syndrome. *Eneuro.* (2017) 4:ENEURO.0380-16.2017. doi: 10.1523/ENEURO.0380-16.2017
52. Pirbhoy PS, Rais M, Lovelace JW, Woodard W, Razak KA, Binder DK, et al. Acute pharmacological inhibition of matrix metalloproteinase-9 activity during development restores perineuronal net formation and normalizes auditory processing in Fmr1 KO mice. *J Neurochem.* (2020) 155:538–58. doi: 10.1111/jnc.15037
53. Berzhanskaya J, Phillips MA, Gorin A, Lai C, Shen J, Colonnese MT. Disrupted cortical state regulation in a rat model of fragile X syndrome. *Cereb Cortex.* (2016) 27:bhv331. doi: 10.1093/cercor/bhv331
54. Jonak CR, Sandhu MS, Assad SA, Barbosa JA, Makhija M, Binder DK. The PDE10A inhibitor TAK-063 reverses sound-evoked EEG abnormalities in a mouse model of fragile X syndrome. *Neurotherapeutics.* (2021). doi: 10.1007/s13311-021-01042-5. [Epub ahead of print].
55. Kulinich AO, Reinhard SM, Rais M, Lovelace JW, Scott V, Binder DK, et al. Beneficial effects of sound exposure on auditory cortex development in a mouse model of fragile X syndrome. *Neurobiol Dis.* (2020) 134:104622. doi: 10.1016/j.nbd.2019.104622
56. Lovelace JW, Wen TH, Reinhard S, Hsu MS, Sidhu H, Ethell IM, et al. Matrix metalloproteinase-9 deletion rescues auditory evoked potential habituation deficit in a mouse model of Fragile X Syndrome. *Neurobiol Dis.* (2016) 89:126–35. doi: 10.1016/j.nbd.2016.02.002

57. Kozono N, Okamura A, Honda S, Matsumoto M, Mihara T. Gamma power abnormalities in a Fmr1-targeted transgenic rat model of fragile X syndrome. *Sci Rep.* (2020) 10:1–9. doi: 10.1038/s41598-020-75893-x
58. Jonak CR, Lovelace JW, Ethell IM, Razak KA, Binder DK. Reusable multielectrode array technique for electroencephalography in awake freely moving mice. *Front Integr Neurosci.* (2018) 12:53. doi: 10.3389/fnint.2018.00053
59. Wen TH, Afroz S, Reinhard SM, Palacios AR, Tapia K, Binder DK, et al. Genetic reduction of matrix metalloproteinase-9 promotes formation of perineuronal nets around parvalbumin-expressing interneurons and normalizes auditory cortex responses in developing Fmr1 knock-out mice. *Cereb Cortex.* (2018) 28:3951–64. doi: 10.1093/cercor/bhx258
60. Rotschafer S, Razak K. Altered auditory processing in a mouse model of fragile X syndrome. *Brain Res.* (2013) 1506:12–24. doi: 10.1016/j.brainres.2013.02.038
61. Gibson JR, Bartley AF, Hays SA, Huber KM. Imbalance of neocortical excitation and inhibition and altered UP states reflect network hyperexcitability in the mouse model of fragile X syndrome. *J Neurophysiol.* (2008) 100:2615–26. doi: 10.1152/jn.90752.2008
62. Patel AB, Hays SA, Bureau I, Huber KM, Gibson JR. A target cell-specific role for presynaptic Fmr1 in regulating glutamate release onto neocortical fast-spiking inhibitory neurons. *J Neurosci.* (2013) 33:2593–604. doi: 10.1523/JNEUROSCI.2447-12.2013
63. Patel AB, Loerwald KW, Huber KM, Gibson JR. Postsynaptic FMRP promotes the pruning of cell-to-cell connections among pyramidal neurons in the L5A neocortical network. *J Neurosci.* (2014) 34:3413–8. doi: 10.1523/JNEUROSCI.2921-13.2014
64. Oswald AMM, Reyes AD. Maturation of intrinsic and synaptic properties of layer 2/3 pyramidal neurons in mouse auditory cortex. *J Neurophysiol.* (2008) 99:2998–3008. doi: 10.1152/jn.01160.2007
65. Oswald AMM, Reyes AD. Development of inhibitory timescales in auditory cortex. *Cereb Cortex.* (2011) 21:1351–61. doi: 10.1093/cercor/bhq214
66. Kim H, Gibboni R, Kirkhart C, Bao S. Impaired critical period plasticity in primary auditory cortex of fragile X model mice. *J Neurosci.* (2013) 33:15686–92. doi: 10.1523/JNEUROSCI.3246-12.2013
67. Yang S, Yang S, Park JS, Kirkwood A, Bao S. Failed stabilization for long-term potentiation in the auditory cortex of FMR1 knockout mice. *PLoS ONE.* (2014) 9:e104691. doi: 10.1371/journal.pone.0104691
68. Wang Y, Sakano H, Beebe K, Brown MR, de Laat R, Bothwell M, et al. Intense and specialized dendritic localization of the fragile X mental retardation protein in binaural brainstem neurons: a comparative study in the alligator, chicken, gerbil, and human. *J Compar Neurol.* (2014) 522:2107–28. doi: 10.1002/cne.23520
69. Zorio DA, Jackson CM, Liu Y, Rubel EW, Wang Y. Cellular distribution of the fragile X mental retardation protein in the mouse brain. *J Compar Neurol.* (2017) 525:818–49. doi: 10.1002/cne.24100
70. McCullagh EA, Rotschafer SE, Auerbach BD, Klug A, Kaczmarek LK, Cramer KS, et al. Mechanisms underlying auditory processing deficits in Fragile X syndrome. *FASEB J.* (2020) 34:3501–18. doi: 10.1096/fj.201902435R
71. Rotschafer SE, Cramer KS. Developmental emergence of phenotypes in the auditory brainstem nuclei of fmr1 knockout mice. *eNeuro.* (2017) 4:ENEURO.0264-17.2017. doi: 10.1523/ENEURO.0264-17.2017
72. Garcia-Pino E, Gessele N, Koch U. Enhanced excitatory connectivity and disturbed sound processing in the auditory brainstem of fragile X mice. *J Neurosci.* (2017) 37:7403–19. doi: 10.1523/JNEUROSCI.2310-16.2017
73. Nguyen AO, Binder DK, Ethell IM, Razak KA. Abnormal development of auditory responses in the inferior colliculus of a mouse model of fragile X syndrome. *J Neurophysiol.* (2020) 123:2101–21. doi: 10.1152/jn.00706.2019
74. Sturm J, Nguyen T, Kandler K. Development of intrinsic connectivity in the central nucleus of the mouse inferior colliculus. *J Neurosci.* (2014) 34:15032–46. doi: 10.1523/JNEUROSCI.2276-14.2014
75. Gonzalez D, Tomasek M, Hays S, Sridhar V, Ammanuel S, Chang CW, et al. Audiogenic seizures in the *Fmr1* knock-out mouse are induced by *Fmr1* deletion in subcortical, VGlut2-expressing excitatory neurons and require deletion in the inferior colliculus. *J Neurosci.* (2019) 39:9852–63. doi: 10.1523/JNEUROSCI.0886-19.2019
76. Goswami S, Cavalier S, Sridhar V, Huber KM, Gibson JR. Local cortical circuit correlates of altered EEG in the mouse model of Fragile X syndrome. *Neurobiol Dis.* (2019) 124:563–72. doi: 10.1016/j.nbd.2019.01.002
77. Selby L, Zhang C, Sun QQ. Major defects in neocortical GABAergic inhibitory circuits in mice lacking the fragile X mental retardation protein. *Neurosci Lett.* (2007) 412:227–32. doi: 10.1016/j.neulet.2006.11.062
78. Goel A, Cantu DA, Guilfoyle J, Chaudhari GR, Newadkar A, Todisco B, et al. Impaired perceptual learning in a mouse model of Fragile X syndrome is mediated by parvalbumin neuron dysfunction and is reversible. *Nat Neurosci.* (2018) 21:1404–11. doi: 10.1038/s41593-018-0231-0
79. Nomura T, Musial TF, Marshall JJ, Zhu Y, Remmers CL, Xu J, et al. Delayed maturation of fast-spiking interneurons is rectified by activation of the TrkB receptor in the mouse model of fragile X syndrome. *J Neurosci.* (2017) 37:11298–310. doi: 10.1523/JNEUROSCI.2893-16.2017
80. Sohal VS, Zhang F, Yizhar O, Deisseroth K. Parvalbumin neurons and gamma rhythms enhance cortical circuit performance. *Nature.* (2009) 459:698–702. doi: 10.1038/nature07991
81. Cardin JA, Carlén M, Meletis K, Knoblich U, Zhang F, Deisseroth K, et al. Driving fast-spiking cells induces gamma rhythm and controls sensory responses. *Nature.* (2009) 459:663–7. doi: 10.1038/nature08002
82. Chen G, Zhang Y, Li X, Zhao X, Ye Q, Lin Y, et al. Distinct inhibitory circuits orchestrate cortical beta and gamma band oscillations. *Neuron.* (2017) 96:1403–18. doi: 10.1016/j.neuron.2017.11.033
83. Khoshkhoo S, Vogt D, Sohal VS. Dynamic, cell-type-specific roles for GABAergic interneurons in a mouse model of optogenetically inducible seizures. *Neuron.* (2017) 93:291–8. doi: 10.1016/j.neuron.2016.11.043
84. Guyon N, Zacharias LR, de Oliveira EF, Kim H, Leite JP, Lopes-Aguiar C, et al. Network asynchrony underlying increased broadband gamma power. *J Neurosci.* (2021) 41:2944–63. doi: 10.1523/JNEUROSCI.2250-20.2021
85. Balmer TS. Perineuronal nets enhance the excitability of fast-spiking neurons. *Eneuro.* (2016) 3:ENEURO.0112-16.2016. doi: 10.1523/ENEURO.0112-16.2016
86. Pizzorusso T, Medini P, Berardi N, Chierzi S, Fawcett JW, Maffei L. Reactivation of ocular dominance plasticity in the adult visual cortex. *Science.* (2002) 298:1248–51. doi: 10.1126/science.1072699
87. Lee HHC, Bernard C, Ye Z, Acampora D, Simeone A, Prochiantz A, et al. Genetic Otx2 mis-localization delays critical period plasticity across brain regions. *Mol Psychiatry.* (2017) 22:680–8. doi: 10.1038/mp.2017.1
88. Lensjø KK, Lepperød ME, Dick G, Hafting T, Fyhn M. Removal of perineuronal nets unlocks juvenile plasticity through network mechanisms of decreased inhibition and increased gamma activity. *J Neurosci.* (2017) 37:1269–83. doi: 10.1523/JNEUROSCI.2504-16.2016
89. Murase S, Lantz CL, Quinlan EM. Light reintroduction after dark exposure reactivates plasticity in adults via perisynaptic activation of MMP-9. *Elife.* (2017) 6:e27345. doi: 10.7554/eLife.27345.025
90. Reinhard SM, Razak K, Ethell IM. A delicate balance: role of MMP-9 in brain development and pathophysiology of neurodevelopmental disorders. *Front Cell Neurosci.* (2015) 9:280. doi: 10.3389/fncel.2015.00280
91. Janusz A, Miłek J, Perycz M, Pacini L, Bagni C, Kaczmarek L, et al. The fragile X mental retardation protein regulates matrix metalloproteinase 9 mRNA at synapses. *J Neurosci.* (2013) 33:18234–41. doi: 10.1523/JNEUROSCI.2207-13.2013
92. Sidhu H, Dansie LE, Hickmott PW, Ethell DW, Ethell IM. Genetic removal of matrix metalloproteinase 9 rescues the symptoms of fragile X syndrome in a mouse model. *J Neurosci.* (2014) 34:9867–79. doi: 10.1523/JNEUROSCI.1162-14.2014
93. Kokash J, Alderson EM, Reinhard SM, Crawford CA, Binder DK, Ethell IM, et al. Genetic reduction of MMP-9 in the Fmr1 KO mouse partially rescues prepulse inhibition of acoustic startle response. *Brain Res.* (2019) 1719:24–9. doi: 10.1016/j.brainres.2019.05.029
94. Gkogkas CG, Khoutorsky A, Cao R, Jafarnejad SM, Prager-Khoutorsky M, Giannakas N, et al. Pharmacogenetic inhibition of eIF4E-dependent Mmp9 mRNA translation reverses fragile X syndrome-like phenotypes. *Cell Rep.* (2014) 9:1742–55. doi: 10.1016/j.celrep.2014.10.064
95. Siller SS, Broadie K. Neural circuit architecture defects in a Drosophila model of Fragile X syndrome are alleviated by minocycline treatment and genetic removal of matrix metalloproteinase. *Dis Model Mech.* (2011) 4:673–85. doi: 10.1242/dmm.008045

96. Dziembowska M, Pretto DI, Janusz A, Kaczmarek L, Leigh MJ, Gabriel N, et al. High MMP 9 activity levels in fragile X syndrome are lowered by minocycline. *Am J Med Genet.* (2013) 161A:1897–903. doi: 10.1002/ajmg.a.36023
97. Paribello C, Tao L, Folino A, Berry-Kravis E, Tranfaglia M, Ethell IM, et al. Open-label add-on treatment trial of minocycline in fragile X syndrome. *BMC Neurol.* (2010) 10:1–9. doi: 10.1186/1471-2377-10-91
98. Leigh MJS, Nguyen DV, Mu Y, Winarni TI, Schneider A, Chechi T, et al. A randomized double-blind, placebo-controlled trial of minocycline in children and adolescents with fragile x syndrome. *J Dev Behav Pediatrics.* (2013) 34:147. doi: 10.1097/DBP.0b013e318287cd17
99. Rotschafer SE, Trujillo MS, Dansie LE, Ethell IM, Razak KA. Minocycline treatment reverses ultrasonic vocalization production deficit in a mouse model of Fragile X Syndrome. *Brain Res.* (2012) 1439:7–14. doi: 10.1016/j.brainres.2011.12.041
100. Toledo MA, Wen TH, Binder DK, Ethell IM, Razak KA. Reversal of ultrasonic vocalization deficits in a mouse model of fragile X syndrome with minocycline treatment or genetic reduction of MMP-9. *Behav Brain Res.* (2019) 372:112068. doi: 10.1016/j.bbr.2019.112068
101. Dansie LE, Phommahaxay K, Okusanya AG, Uwadia J, Huang M, Rotschafer SE, et al. Long-lasting effects of minocycline on behavior in young but not adult Fragile X mice. *Neuroscience.* (2013) 246:186–98. doi: 10.1016/j.neuroscience.2013.04.058
102. Rotschafer SE, Razak KA. Auditory processing in fragile x syndrome. *Front Cell Neurosci.* (2014) 8:19. doi: 10.3389/fncel.2014.00019
103. Wadell MP, Hagerman J, Hessl RRD. Fragile X syndrome: psychiatric manifestations, assessment and emerging therapies. *Curr Psychiatry Rev.* (2013) 9:53–8. doi: 10.2174/1573400511309010008
104. Yamasue H, Aran A, Berry-Kravis E. Emerging pharmacological therapies in fragile X syndrome and autism. *Curr Opin Neurol.* (2019) 32:635–40. doi: 10.1097/WCO.0000000000000703
105. Berry-Kravis EM, Lindemann L, Jønch AE, Apostol G, Bear MF, Carpenter RL, et al. Drug development for neurodevelopmental disorders: lessons learned from fragile X syndrome. *Nat Rev Drug Discov.* (2018) 17:280–99. doi: 10.1038/nrd.2017.221
106. Berry-Kravis EM, Harnett MD, Reines SA, Reese MA, Ethridge LE, Outterson AH, et al. Inhibition of phosphodiesterase-4D in adults with fragile X syndrome: a randomized, placebo-controlled, phase 2 clinical trial. *Nat Med.* (2021) 27:862–70. doi: 10.1038/s41591-021-01321-w
107. Bear MF, Huber KM, Warren ST. The mGluR theory of fragile X mental retardation. *Trends Neurosci.* (2004) 27:370–7. doi: 10.1016/j.tins.2004.04.009
108. Mascio G, Bucci D, Notartomaso S, Liberatore F, Antenucci N, Scarselli P, et al. Perineuronal nets are under the control of type-5 metabotropic glutamate receptors in the developing somatosensory cortex. *Transl Psychiatry.* (2021) 11:1–13. doi: 10.1038/s41398-021-01210-3
109. Russo NM, Skoe E, Trommer B, Nicol T, Zecker S, Bradlow A, et al. Deficient brainstem encoding of pitch in children with autism spectrum disorders. *Clin Neurophysiol.* (2008) 119:1720–31. doi: 10.1016/j.clinph.2008.01.108
110. Wong PC, Skoe E, Russo NM, Dees T, Kraus N. Musical experience shapes human brainstem encoding of linguistic pitch patterns. *Nat Neurosci.* (2007) 10:420–2. doi: 10.1038/nn1872
111. Brager DH, Akhavan AR, Johnston D. Impaired dendritic expression and plasticity of h-channels in the fmr1–/y mouse model of fragile X syndrome. *Cell Rep.* (2012) 1:225–33. doi: 10.1016/j.celrep.2012.02.002
112. Zhang Z, Marro SG, Zhang Y, Arendt KL, Patzke C, Zhou B, et al. The fragile X mutation impairs homeostatic plasticity in human neurons by blocking synaptic retinoic acid signaling. *Sci Transl Med.* (2018) 10:eaar4338. doi: 10.1126/scitranslmed.aar4338
113. Bülow P, Murphy TJ, Bassell GJ, Wenner P. Homeostatic intrinsic plasticity is functionally altered in Fmr1 KO cortical neurons. *Cell Rep.* (2019) 26:1378–88. doi: 10.1016/j.celrep.2019.01.035
114. Hampson DR, Hooper AW, Niibori Y. The application of Adeno-associated viral vector gene therapy to the treatment of fragile X syndrome. *Brain Sci.* (2019) 9:32. doi: 10.3390/brainsci9020032
115. Shitik EM, Velmiskina AA, Dolskiy AA, Yudkin DV. Reactivation of FMR1 gene expression is a promising strategy for fragile X syndrome therapy. *Gene Ther.* (2020) 27:247–53. doi: 10.1038/s41434-020-0141-0
116. Siegel JJ, Chitwood RA, Ding JM, Payne C, Taylor W, Gray R, et al. Prefrontal cortex dysfunction in fragile x mice depends on the continued absence of fragile x mental retardation protein in the adult brain. *J Neurosci.* (2017) 37:7305–17. doi: 10.1523/JNEUROSCI.0571-17.2017
117. Guy J, Gan J, Selfridge J, Cobb S, Bird A. Reversal of neurological defects in a mouse model of Rett syndrome. *Science.* (2007) 315:1143–7. doi: 10.1126/science.1138389
118. Silva-Santos S, Van Woerden GM, Bruinsma CF, Mientjes E, Jolfaei MA, Distel B, et al. Ube3a reinstatement identifies distinct developmental windows in a murine Angelman syndrome model. *J Clin Invest.* (2015) 125:2069–76. doi: 10.1172/JCI80554
119. Gu B, Carstens KE, Judson MC, Dalton KA, Rougié M, Clark EP, et al. Ube3a reinstatement mitigates epileptogenesis in Angelman syndrome model mice. *J Clin Invest.* (2019) 129:163–8. doi: 10.1172/JCI120816

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Neuroimaging Insight Into Fragile X-Associated Neuropsychiatric Disorders: Literature Review

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FMR1 premutation is defined by 55–200 CGG repeats in the Fragile X Mental Retardation 1 (*FMR1*) gene. *FMR1* premutation carriers are at risk of developing a neurodegenerative disease called fragile X-associated tremor/ataxia syndrome (FXTAS) and Fragile X-associated primary ovarian insufficiency (FXPOI) in adulthood. In the last years an increasingly board spectrum of clinical manifestations including psychiatric disorders have been described as occurring at a greater frequency among *FMR1* premutation carriers. Herein, we reviewed the neuroimaging findings reported in relation with psychiatric symptomatology in adult *FMR1* premutation carriers. A structured electronic literature search was conducted on *FMR1* premutation and neuroimaging yielding a total of 3,229 articles examined. Of these, 7 articles were analyzed and are included in this review. The results showed that the main radiological findings among adult *FMR1* premutation carriers presenting neuropsychiatric disorders were found on the amygdala and hippocampus, being the functional abnormalities more consistent and the volumetric changes more inconsistent among studies. From a molecular perspective, CGG repeat size, *FMR1* mRNA and FMRP levels have been investigated in relation with the neuroimaging findings. Based on the published results, FMRP might play a key role in the pathophysiology of the psychiatric symptoms described among *FMR1* premutation carriers. However, additional studies including further probes of brain function and a broader scope of psychiatric symptom measurement are required in order to obtain a comprehensive landscape of the neuropsychiatric phenotype associated with the *FMR1* premutation.

Keywords: FXAND, neuropathology, *FMR1* premutation, neuroimaging, functional studies

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INTRODUCTION

Fragile X premutation carriers is defined by 55–200 CGG repeats in the Fragile X Mental Retardation 1 (*FMR1*) gene, whilst the full mutation is caused by >200 CGG repeats. Two major conditions associated with the *FMR1* premutation have been well-established: the fragile X-associated Tremor/Ataxia Syndrome (FXTAS) and the fragile X-associated Primary Ovarian

Insufficiency (FXPOI) [reviewed in (1)] FXTAS is a late onset neurodegenerative condition, and it is seen in around 40% of male premutation carriers and 16% of females (2). FXPOI is characterized by menopause before age 40 and it is seen in around 20% of women with the *FMR1* premutation (3). Nevertheless, a broader clinical spectrum of symptoms, including psychiatric, sleep, and autoimmune conditions, has been described among *FMR1* permutation carriers (4). Although the extent of all this group of conditions needs further delineation, in order to bring recognition to these problems, different names were proposed. Hagerman et al., proposed fragile X-associated Neuropsychiatric Disorders (FXAND) and the European Fragile X Network (EFXN) proposed to name them Fragile X-associated Neuropsychiatric Conditions (FXANC), and Fragile X Various Associated Conditions (FXVAC) to cover other physical conditions associated with the *FMR1* premutation (51).

FMR1 premutation main neuropsychiatric disorders in adults include anxiety and depression and, to a lesser extent, obsessive compulsive disorder (OCD), attention-deficit/hyperactivity disorder (ADHD), and substance abuse. Although most prior work has been performed examining different neuropsychiatric aspects in separate study samples, neuroimaging studies have depicted structural, functional, and connectivity changes within the brain associated to neuropsychiatric disorders. In regard to *FMR1* premutation this relationship has been addressed, although with different approaches and with different population cohorts. Furthermore, a correlation on how neurostructural and neurofunctional effects might be associated with molecular aspects of the *FMR1* premutation and psychiatric symptomatology has also been reported with varying results. This review focuses on the neuroimaging findings associated with neuropsychiatric disorders in adult *FMR1* premutation carriers. Due to the wide clinical spectrum of neurological symptoms associated with the *FMR1* premutation, including motor and cognitive impairment, executive and memory deficits, and psychiatric symptoms, we believe that a review focusing on the neuropathology, molecular underpinnings, and neuroimaging associated with neuropsychiatric findings could help to untangle the complex physiopathology associated with the *FMR1* premutation.

METHODS

Search Strategy

A review of the literature was conducted. PubMed, Web of Science, Pschynfo and Cochrane Central Register of Controlled Trials were searched for eligible studies from 2000 to April 2021. Search terms are listed in Research Algorithm (**Supplementary Table 1**) where the complete search strategy is displayed. Inclusion criteria included *FMR1* premutation carriers, both genders, from 18 to 99 years old and all ethnicities. As exclusion criteria, the search was only focused on pure psychiatric symptoms and therefore motor and cognitive impairments, executive function, or memory deficits were not considered.

Study Eligibility Criteria

Eligible qualitative studies were those that matched neuroimaging findings with psychiatric symptomatology in *FMR1* premutation carriers. The type of studies selected were those of any design published in peer-reviewed academic journals with abstract available. Conference proceedings, theses, case reports and case series were excluded, and review articles, non-English articles, and studies on animal models were not considered (for a graphical summary of the selection procedure, see **Figure 1**).

After establishing and applying the inclusion criteria, two researchers (A. EM and L.RR) separately read the titles of the papers retrieved in the search and examined the structured abstract of the selected articles. Seven studies that described the relationship between neuroimaging and neuropsychiatric findings in adult *FMR1* premutation were selected. All potential differences in interpretation between the reviewers were discussed to ensure that all the articles reviewed presented a satisfactory level of evidence.

This study is a review of previously published data and, as such, does not require ethics approval. The data were not used for any purpose other than those of the original study, and no new data were collected.

RESULTS

Synthesis of Qualitative Studies

A total of 3,229 studies were identified from the initial search. After de-duplication and initial screening, 1,933 full-text articles were assessed for eligibility and 7 were included in this review (**Figure 1**, **Table 1**) (5–11).

Represented in these manuscripts were views from 413 study participants (244 *FMR1* premutation carriers and 169 control individuals) from 2 countries (UK, USA). Participants included adult men and women ranging in age from 18 to over 79 years. *FMR1* premutation carriers were recruited through screening of pedigrees of probands with FXS with a CGG repeat size ranging from 55 CGGs to 199 CGGs. All subjects who participated in Koldewyn et al. (6) also participated in the Hessl et al. (5) study and, therefore, were counted only once. Such relation was not mentioned in the rest of the studies. One study (11) included 3 *FMR1* premutation mosaic participants and 1 intermediate allele participant within the premutation group and, thus, was not considered when evaluating the CGG range (**Table 2**).

All but one of the seven studies were performed in male population, whereas only one included both male and female permutation carriers. Four of them used functional magnetic resonance imaging (fMRI), five of them used volumetric measures and one used voxel-based morphometry. It is important to note that comorbid medical and psychiatric conditions, such as the mood of participants when performing tasks, were not considered, or at least not mentioned in any of the reviewed studies. Moreover, some of the participants enrolled in the studies were medicated with different drugs (**Table 2**), which might act as a potential confounding factor, and finally had an effect on the neuroimaging findings.

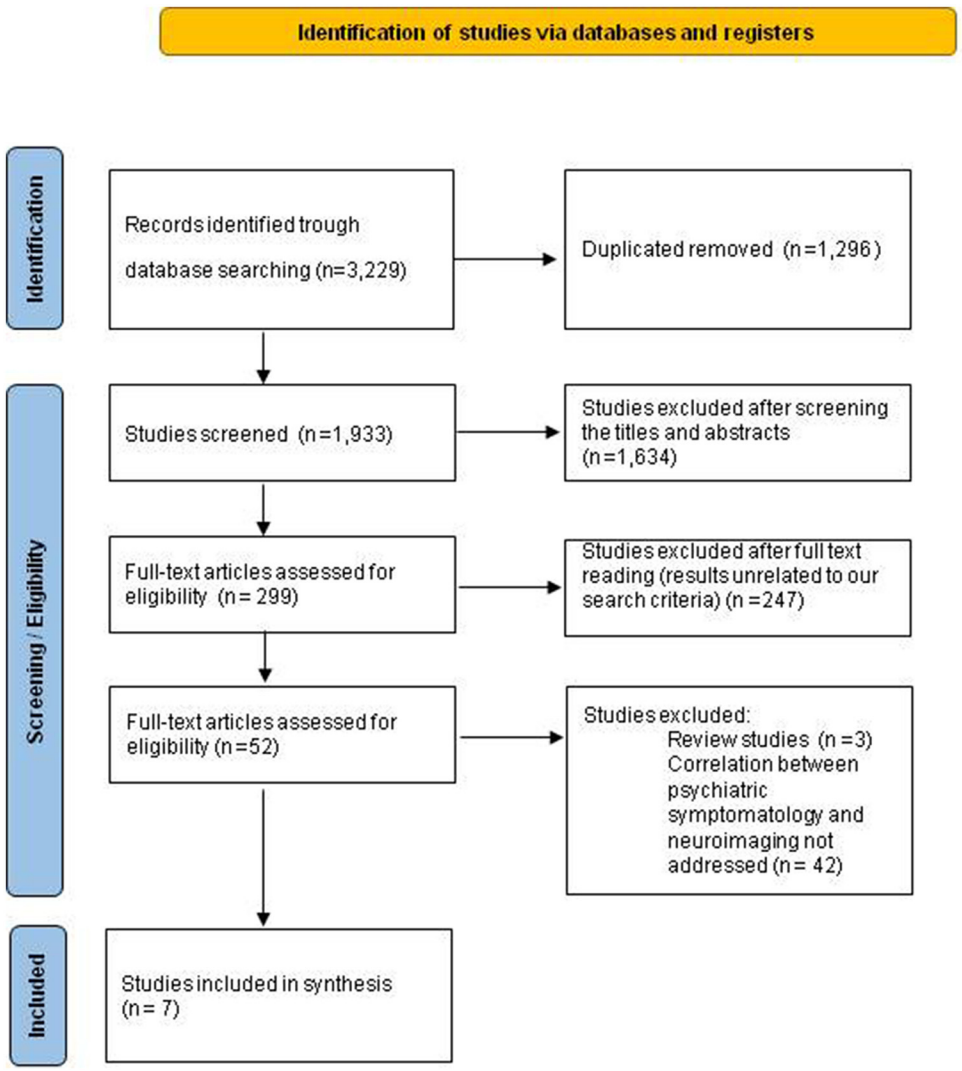

FIGURE 1 | Diagram for identification and selection of studies.

The synthesis of the 7 neuroimaging studies identified the amygdala and the hippocampus as the two major brain areas involved with FXAND. **Table 3** summarizes results reported in the 7 studies.

Amygdala

The relationship between both the amygdala function and volume with psychological symptoms has been evaluated in several studies.

Regarding the amygdala function, male premutation carriers without FXTAS have been found to have decreased amygdala activation to emotional inputs which correlate with an increase of psychological symptoms. In 2007, Hessl et al. (5) described a negative correlation between psychological symptoms and amygdala activation compared to controls during an fMRI task consistent in passively viewing fearful vs. scrambled faces. Moreover, they also found that *FMR1* premutation participants had decreased potentiation of the eye blink startle reflex to fearful faces, which is an indirect evidence of reduced amygdale activation, and diminished skin conductance response during a brief social stressor; evidencing reduced sympathetic activation. Similarly, Hessl et al. (9) described a negative correlation between higher ratings of autism spectrum symptoms and reduced left amygdala activation during an emotion processing fMRI task.

The reduced amygdala activation in *FMR1* premutation carriers has been associated with abnormal elevation of *FMR1* mRNA (5) and in particular, with reduced FMRP levels (9).

As for the amygdala volume and its correlation with psychological symptoms, results are controversial. While some studies did not find differences between groups in the amygdala volume and psychological symptoms (measured on the SCL-90-R) or cognitive ability (based on full scale IQ) (5, 10), others reported higher ratings of autism spectrum symptoms correlated with smaller bilateral amygdala volume (9). Additionally,

TABLE 1 | Summary of the reviewed studies.

Author and Year	Participants	Cognitive test	Psychological test	Molecular measures	Imaging (MRI)	Other
Hessl et al. (5)	12 *FMR1* premutation males without FXTAS 13 male controls	WAIS-III	SCL-90-R	CGG repeat size *FMR1* mRNA	Amygdala volumes Total cranial volume Total brain volume fMRI face processing task	Eye blink response to fear-potentiated startle (EMG activity of orbicularis oculi) Skin conductance during social greeting (electrodermal electrodes)
Koldewyn et al. (6)	11 *FMR1* premutation males without FXTAS 11 male controls	WAIS-III	SCL-90-R	CGG repeat size *FMR1* mRNA	fMRI recall task Total brain volume Hippocampal volume	-
Adams et al. (7)	16 *FMR1* premutation female with FXTAS 17 *FMR1* premutation female without FXTAS 8 female controls 34 *FMR1* premutation male with FXTAS 21 *FMR1* premutation male without FXTAS 30 male controls	WAIS-III	SCL-90-R	CGG repeat size *FMR1* mRNA methylation status activation ratio	Total brain volume Hippocampal volume	FXTAS clinical staging
Hashimoto et al. (8)	31 *FMR1* premutation male with FXTAS 24 *FMR1* premutation male without FXTAS 28 male controls	Behavioral Dyscontrol Scale 2 Working Memory Score of the WAIS-III	SCL-90-R	CGG repeat size *FMR1* mRNA	Voxel-based morphometry analysis	FXTAS clinical staging
Hessl et al. (9)	23 *FMR1* premutation male without FXTAS 25 male controls	WAIS-III	Autism Diagnostic Observation Schedule Module 4 SRS (adult version)	CGG repeat size *FMR1* mRNA methylation status FMRP levels	Amygdala volume Total cerebral volume fMRI targeting the amygdala with an emotion processing task and concurrent infra-red eye tracking	-
Selmeczy et al. (10)	49 *FMR1* premutation male without FXTAS 48 male controls	WAIS-III	SCL-90-R	CGG repeat size *FMR1* mRNA	Amygdala volume Total cerebral volume	-
Brown et al. (11)	17 *FMR1* premutation male without FXTAS 17 male controls	KBIT	SCL-90-R AQ EQ Ekman Faces Test (version 1.0)	CGG repeat size	Emotional processing fMRI task to examine the response to a change in emotional arousal	-

SCL-90-R, Symptom Checklist-90—Revised; WAIS-III, Wechsler Adult Intelligence Scale (Third Edition); KBIT, Kaufman Brief Intelligence Test (Second Edition); AQ, Autism Spectrum Quotient; EQ, Empathy Quotient; SRS, Social Responsiveness Scale.

TABLE 2 | Study participant characteristics.

Study (Country)	Control population					*FMR1* premutation				
	Number of participants	**Age (years old)**	**Ethnicity**	**CGGs repeat size**	**Medical history**	**Number of participants**	**Age (years old)**	**Ethnicity**	**CGGs repeat size**	**Medical history**
Hessl et al. (5) (USA)	*N* = 11 (all men)	26–55	2 Hispanic 1 East Indian 8 Caucasian	17–32	Neurological examination normal 1 participant on venlafaxine and buproprion	*N* = 11 (all men)	28–56	2 Hispanic 9 Caucasian	57–166	Neurological examination normal 2 participants on amitriptyline
Koldewyn et al. (6) (USA)	Same participants as Hessl et al. (5)									
Adams et al. (7) (USA)	*N* = 38 (8 females, 30 males)	Female: 50.63 ± 11.43 (mean ± SD) Male: 57.20 ± 14.12 (mean ± SD)	NA	Female: 32 ± 6.89 (mean ± SD) Male: 28.42 ± 4.82 (mean ± SD)	No significant difference in psychotropic medication taken	*N* = 88 (16 female with FXTAS, 17 Female without FXTAS, 34 male with FXTAS, 21 male without FXTAS)	Female with FXTAS: 57.50 ± 12.46 (mean ± SD) Female without FXTAS: 44.94 ± 11.23 (mean ± SD) Male with FXTAS: 66.44 ± 6.77 (mean ± SD) Male without FXTAS: 52.38 ± 12.11 (mean ± SD)	NA	Female with FXTAS: 95.19 ± 14.18 (mean ± SD) Female without FXTAS: 96.40 ± 20.17 (mean ± SD) Male with FXTAS: 92.76 ± 18.86 (mean ± SD) Male without FXTAS: 88.52 ± 32.33 (mean ± SD)	75% of females with FXTAS and 41.2% of males with FXTAS on psychotropic medication
Hashimoto et al. (8) (USA)	*N* = 28 (all men)	40–79	NA	17–34	Neurological examination normal	*N* = 55 (31 male with FXTAS, 24 male without FXTAS)	Male with FXTAS: 47–79 Male without FXTAS: 41–78	NA	Male with FXTAS: 59–130 Male without FXTAS: 55–166	NA
Hessl et al. (9) (USA)	*N* = 25 (all men)	30.12 ± 7. 5 (mean ± SD)	68.5% Caucasian	19–33	Neurological examination normal 33.3% Psychoactive medication	*N* = 23 (all men)	32.95 ± 8.89 (mean ± SD)	82.6% Caucasian	55–199	Neurological examination normal 28.6% Psychoactive medication
Selmeczy et al. (10) (USA)	*N* = 48 (all men)	19–76	NA	17–42	Neurological examination normal	*N* = 49 (all men)	18–78	NA	55–199	Neurological examination normal
Brown et al. (11) (UK)	*N* = 17 (all men)	24–68	NA	19–40	Neurological examination normal	*N* = 17 (all men)	24–68	NA	58–185 3 premutation mosaic (150 ± 5; ∼200 ± 10), (133, 156; 198 ± 10), (148 ± 5; ∼200) 1 Intermediate allele (50 CGGs)	Neurological examination normal

Hashimoto et al. (8) demonstrated that increased levels of obsessive–compulsiveness and depression in male premutation carriers was associated with gray matter loss in the left amygdala evaluated by voxel-based morphometry. A plausible explanation for these discordant results might be the different neuroimaging methods used in each study. Whereas, Selmeczy et al. (10) used both 1.5 and 3.0 Tesla MRI, the study conducted by Hessel et al. (9) only used a 3.0 T structural imaging.

Interestingly, even though Selmeczy et al. (10) found no difference between groups in the amygdala volume, a significant

TABLE 3 | Summary of the psychological, neuroimaging and molecular findings reported in *FMR1* premutation carriers compared to control population in the papers reviewed.

Study	Psychological findings	Neuroimaging findings	Molecular findings
Hessl et al. (5)	Diminished autonomic responsivity to social–emotional stimuli	Diminished amygdala activation No differences in amygdala volume	Positive correlation with increased *FMR1* mRNA levels
Koldewyn et al. (6)	Significantly worse performance on an immediate memory recall test but not on the in-scanner recall task 24 h later	Reduced left hippocampal activation and increased right parietal activation No differences in hippocampal volume	Positive correlation with increased *FMR1* mRNA levels No significant correlation with the CGG repeat number
Adams et al. (7)	Increased psychological symptoms in *FMR1* premutation carriers with FXTAS (obsessive–compulsive behaviors, somatization and anxiety)	Significant negative correlations between hippocampal volume and psychological symptoms	Negative correlation between CGG repeat size and total and left hippocampal volume in males with FXTAS. Negative correlation between CGG repeat length and right hippocampal volume in females with FXTAS Positive correlation between *FMR1* mRNA levels and left hippocampal volume in female carriers without FXTAS.
Hashimoto et al. (8)	Increased levels of obsessive–compulsiveness and depression Poor working memory performance	Gray matter loss in the left amygdala Decreased gray matter in the left inferior frontal cortex and anterior cingulate cortex	Significant negative effect of CGG repeat size on gray matter density in the dorsomedial frontal regions
Hessl et al. (9)	Higher ratings of communication and reciprocal social behavior symptoms of autism spectrum disorder (althought not significant)	Significantly smaller left and right amygdala volume during an emotion-matching task Reduced right amygdala activation during the task	Reduced FMRP as the primary factor involved in alterations of brain activity and behavior
Selmeczy et al. (10)	No difference on the global severity index score of the SCL-90-R	No significant differences in amygdala volume	Significant negative correlation between amygdala volume and CGG repeat expansions was found in the lower, but not in the higher, range of CGG repeat expansions
Brown et al. (11)	Significantly worse symptoms of neuropsychiatric symptomatology of obsessive-compulsiveness, anxiety, global psychiatric severity and positive symptom distress levels Higher levels of autistic traits Impaired facial emotion recognition	Significantly lower activation at the bilateral superior parietal lobes, bilateral Brodmann Area (BA) 17 (primary visual cortex), right intraparietal area and right BA18 (visual association area) when comparing high and low arousal conditions	Not examined

negative correlation was found between amygdala volume and the lower range of CGG repeat expansion (CGG≥55 and <85), but not in the higher range of CGG repeat expansion (CGG≥85). This observation raises the intriguing possibility that different molecular mechanisms could be affecting the brain structure, and potentially the function, in *FMR1* premutation carriers depending on the CGG repeat expansion size.

Hippocampus

The relationship between psychological symptoms and hippocampal function and volume in *FMR1* premutation carriers has also been examined.

Reduced hippocampal activation during an fMRI memory recall task has been associated to psychiatric symptomology in *FMR1* premutation male carriers without FXTAS (6). Moreover, this reduction was found in association with parietal over-activation, which might be a feedback effect to compensate the decreased hippocampal involvement, and abnormal elevation of *FMR1* mRNA.

A hippocampal volume effect has been associated with *FMR1* premutation, albeit some inconsistent findings. While Jäkälä et al. (12) found reduced volumes; Loesch et al. (13) described increased volumes in this region in premutation carriers. On the other hand, Koldewyn et al. (6) did not find hippocampal volume effects associated with the premutation. The lack of consistent findings between these three studies may be due to specific cohort effects, especially if these cohorts included participants with and without FXTAS.

To our knowledge, only two papers explored the relationship between psychological measures and hippocampal size. While Koldewyn et al. (6) did not find hippocampal volume differences, Adams et al. (7) found a significant negative correlation between total hippocampal volume and anxiety in female carriers with and without FXTAS. This association seemed to be mainly driven by the right hippocampus since correlations were stronger. The association in male permutation carriers was weaker and was only significant for one of the psychological problems assessed (paranoid ideation). Furthermore, they found a negative correlation

between CGG repeat size and total and left hippocampal volume in males with FXTAS and a similar correlation with CGG repeat length and right hippocampal volume in females with FXTAS (7).

Other Findings

The selected studies also showed other structural findings in *FMR1* premutation carriers, even though there was no correlation with psychological symptoms. First, total brain volume has been found to be significantly decreased in older permutation carriers (13). Secondly, gray matter loss has also been examined in several studies: all of them evidencing reduced density in several brain regions in the permutation group. In this regard, Hashimoto et al. (8) reported significant gray matter loss in medial temporal lobe structures and cerebellar areas, such as the vermis lobule VII, and the cerebellar hemisphere lobule IX, as well as in multiple regions outside the cerebellum that have been related with several psychiatric conditions. Although significant loss was found when comparing the permutation group with controls, the correlation analysis failed to show significant association between all these areas and psychiatric problems in *FMR1* premutation carriers. Finally, Brown et al. (11) found that permutation carriers exhibited significantly lower BOLD activation compared to controls at the bilateral superior parietal lobe, bilateral Brodmann Area (BA) 17 (V1) (primary visual cortex), right intraparietal area, and right BA18 (V2) (visual association area), when comparing high and low arousal conditions. However, no correlations were found between more psychiatric symptoms and higher levels of autistic traits observed in carriers and BOLD activation at the emotional processing fMRI task.

Study Limitation

Overall, the studies herein reviewed provided valuable neuroimaging data of brain abnormalities in *FMR1* premutation carriers related to neuropsychiatric disorders. However, the majority of them were conducted on small sample sizes of groups, which might have limited detection of true significances. Moreover, some of them included *FMR1* premutation carriers with FXTAS which might also influence significant results. In addition, the neuroimaging methods used were different, which makes it difficult to compare results. It should also be noted that fMRI is a complex technique that can be influenced by many factors such as the paradigm design (the manner of stimulating the brain in order to obtain meaningful information), magnetic field strength, MRI acquisition parameter and subject collaboration. Furthermore, the parameters that have an influence in blood flow and oxygenation have an impact on fMRI signal and, overall, fMRI has a challenging data interpretation (14). Finally, there are several variables that have to be taken into account as potential confounding factors in all the studies reviewed. Aspects such as comorbid medical conditions, medication taken by the participants (psychotropic or psychoactive) or unmeasured (unobserved) factors, such as the mood or the stress of participants, could have influenced the results. The reviewed studies were aware of these aspects and tried to minimize their effect by matching *FMR1* premutation and control groups, although this was not always possible.

DISCUSSION

FMR1 premutation carriers are at risk of developing an adult-onset neurodegenerative disorder named FXTAS (20). In addition, several studies reveal that young *FMR1* premutation carriers are at increased risk for psychiatric conditions, memory problems and executive deficits (15–18). Indeed, brain function is also affected by *FMR1* premutation status in relatively young premutation carriers without FXTAS who demonstrate no overt neurological symptoms (5, 6, 9, 11). Contrary to movement-related neurodegeneration, which increases over time, emotional symptoms seem to be consistent over the lifespan in *FMR1* premutation carriers; suggesting a neurodevelopmental origin, different from the neurodegeneration seen in FXTAS (7, 11, 19).

FMR1 premutation carriers have elevated levels of *FMR1* mRNA and can also have some degree of FMR1 protein (FMRP) deficiency, mainly at the high end of the *FMR1* premutation range (20). FMRP is an RNA binding protein that regulates the translation of many gene products and has been implicated in dendritic maturation and in the formation of axons and myelin (21–23). In fact, FMRP activity is regulated in response to neuronal activity, and is an important mediator of synapse development, synaptic plasticity, learning and memory (reviewed in (52)). The mRNA targets of FMRP have received additional attention due to their enrichment for genes harboring risk to psychiatric disorders (24). Recently, Clifton et al. (52) has reported that a substantial proportion of FMRP targets have functions related to synaptic activity, anatomy or development. The association between synaptic plasticity and psychiatric disorders has been well-established with several genetic and functional studies describing the relevance of imbalanced of excitation and inhibition (25). FMRP levels have been reported to be reduced in *FMR1* premutation brains of a mouse model (26), as well as in patients with psychiatric disorders such as autism, schizophrenia, bipolar disorder, and major depressive disorder (27). Whilst some studies had found a relationship between *FMR1* mRNA levels and psychiatric symptomology or brain function in *FMR1* premutation carriers (5, 6, 28), others have pointed out a stronger relationship with reduced FMRP levels (9). Taking into consideration the importance of FMRP in normal neurodevelopment and its association with psychiatric disorders, there is the possibility that moderate reductions in FMRP levels could play a role in the behavioral dysfunction seen in *FMR1* premutation carriers.

Both structural and functional changes in the hippocampus and amygdala have been found to be altered in *FMR1* premutation carriers and some studies have proved a relationship between such changes and psychiatric symptomatology (5–9). However, while functional changes have been consistently reported (5, 6, 9, 11), volumetric measures showed some inconsistent results, with some studies showing increase, decrease, or no significant differences between hippocampal

and amygdala volumes or voxel density in *FMR1* premutation carriers (6, 10, 12, 13, 29–31). The lack of consistent findings between studies may be due to a cohort effect either in the size, the inclusion of participants with and without FXTAS, gender of participants or differences in the CGG repeat size. Moreover, technical aspects such as the volumetric techniques used, the image quality or segmentation technique followed might also contribute to explain discrepancies. However, and in consonance, findings of amygdala and hippocampal volumes in mood disorders such as depression or anxiety in non *FMR1* premutation carriers have also been conflicting, with some studies reporting positive, negative and no associations (32–46).

Although an association between the above described structural and functional changes and molecular aspects of the *FMR1* pemutation carriers has been proven in some of the studies reported (**Table 3**), there is still need to better define them. What does seem certain, and evidence points to it, is that the limbic system is a brain structure particularly susceptible to RNA toxicity. During normal fetal development, the hippocampus is one of the areas in which *FMR1* transcription is the highest (47) and in adult human brain, the hippocampus demonstrates one of the highest expression rates of *FMR1* mRNA (48). Similarly, *FMR1* mRNA levels are disproportionately increased in the amygdala of *FMR1* premutation carriers (48, 49). Moreover, in post-mortem brain studies of male *FMR1* premutation carriers with FXTAS, it has been shown that, compared to other brain areas, the hippocampi harbors high density of intranuclear inclusions (22, 23). Additionally, the knock-in mouse model of the *FMR1* premutation showed a significantly reduced FMRP expression in several brain regions, including the hippocampus (50).

Future studies with larger and more homogeneous sample size are needed in order to increase statistical power and validate such findings. Furthermore, longitudinal studies will be needed to evaluate progression of the neuroimaging and clinical findings. In addition, looking for modifying factors, either predisposing or protective factors, able to modulate neuroimaging, and clinical symptomatology is a key point for the knowledge and understanding of the disease. It is also crucial to clarify the metabolic causes of brain toxicity and to identify early presymptomatic brain changes that precede, but which are ultimately associated with neuropsychiatry disorders. Finally, further investigations that include quantitative measurements of molecular changes will be of great interest in order to clarify the relative roles of increased *FMR1* mRNA and FMRP protein changes in the Fragile X-associated phenotypes.

Overall this review would like to encourage all *FMR1* research groups furthering investigating the neuropsychiatric involvement in *FMR1* premutation with testing other brain systems, with additional probes of brain function and a broader scope of psychiatric symptom measurement. The combination of all these missing data would help to obtain a comprehensive landscape of the neuropsychiatric phenotype associated with the *FMR1* premutation

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Files**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.728952/full#supplementary-material

REFERENCES

1. Mila M, Alvarez-Mora MI, Madrigal I, Rodriguez-Revenga L. Fragile X syndrome: an overview and update of the FMR1 gene. *Clin Genet.* (2018) 93:197–205. doi: 10.1111/cge.13075
2. Rodriguez-Revenga L, Madrigal I, Pagonabarraga J, Xuncl,à M., Badenas C, Kulisevsky J, et al. Penetrance of FMR1 premutation associated pathologies in fragile X syndrome families. *Eur J Human Genet.* (2009) 17:1359–62. doi: 10.1038/ejhg.2009.51
3. Sherman SL. Premature ovarian failure in the fragile X syndrome. *Am J Med Genet.* (2000) 97:189–94. doi: 10.1002/1096-8628(200023)97:3<189::AID-AJMG1036>3.0.CO;2-J
4. Hagerman RJ, Protic D, Rajaratnam A, Salcedo-Arellano MJ, Aydin EY, Schneider A. Fragile X-Associated Neuropsychiatric Disorders (FXAND). *Front Psychiatry.* (2018) 9:564. doi: 10.3389/fpsyt.2018.00564
5. Hessl D, Rivera S, Koldewyn K, Cordeiro L, Adams J, Tassone F, et al. Amygdala dysfunction in men with the fragile X premutation. *Brain.* (2007) 130:404–16. doi: 10.1093/brain/awl338
6. Koldewyn K, Hessl D, Adams J, Tassone F, Hagerman PJ, Hagerman RJ, et al. Reduced hippocampal activation during recall is associated with elevated FMR1 mRNA and psychiatric symptoms in men with the Fragile X Premutation. *Brain Imaging Behav.* (2008) 2:105–16. doi: 10.1007/s11682-008-9020-9
7. Adams PE, Adams JS, Nguyen DV, Hessl D, Brunberg JA, Tassone F, et al. Psychological symptoms correlate with reduced hippocampal volume in fragile X premutation carriers. *Am J Med Genet B Neuropsychiatr Genet.* (2010) 53B:775–85. doi: 10.1002/ajmg.b.31046
8. Hashimoto R, Javan AK, Tassone F, Hagerman RJ, Rivera SM. A voxel-based morphometry study of grey matter loss in fragile X-associated tremor/ataxia syndrome. *Brain.* (2011) 134:863–78. doi: 10.1093/brain/awq368

9. Hessl D, Wang JM, Schneider A, Koldewyn K, Le L, Iwahashi C, et al. Decreased fragile X mental retardation protein expression underlies amygdala dysfunction in carriers of the fragile X premutation. *Biol Psychiatry.* (2011) 70:859–65. doi: 10.1016/j.biopsych.2011.05.033
10. Selmeczy D, Koldewyn K, Wang JM, Lee A, Harvey D, Hessl DR, et al. Investigation of amygdala volume in men with the fragile X premutation. *Brain Imaging Behav.* (2011) 5:285–94. doi: 10.1007/s11682-011-9132-5
11. Brown SSG, Whalley HC, Kind PC, Stanfield AC. Decreased functional brain response to emotional arousal and increased psychiatric symptomology in FMR1 premutation carriers. *Psychiatry Res Neuroimaging.* (2019) 285:9–17. doi: 10.1016/j.pscychresns.2019.01.011
12. Jäkälä P., Hänninen T, Ryynänen M, Laakso M, Partanen K, Mannermaa A, et al. Fragile-X: neuropsychological test performance, CGG triplet repeat lengths, hippocampal volumes. *J Clin Invest.* (1997) 100:331–8. doi: 10.1172/JCI119538
13. Loesch DZ, Litewka L, Brotchie P, Huggins RM, Tassone F, Cook M. Magnetic resonance imaging study in older fragile X premutation male carriers. *Ann Neurol.* (2005) 58:326–30. doi: 10.1002/ana.20542
14. Specht K. Current challenges in translational and clinical fMRI and future directions. *Front Psychiatry.* (2020) 10:924. doi: 10.3389/fpsyt.2019.00924
15. Aziz M, Stathopulu E, Callias M, Taylor C, Turk J, Oostra B, et al. Clinical features of boys with fragile X premutations and intermediate alleles. *Am J Med Genet B Neuropsychiatr Genet.* (2003) 121B:119–27. doi: 10.1002/ajmg.b.20030
16. Farzin F, Perry H, Hessl D, Loesch D, Cohen J, Bacalman S, et al. Autism spectrum disorders and attention-deficit/hyperactivity disorder in boys with the fragile X premutation. *J Dev Behav Pediatr.* (2006) 27:S137–44. doi: 10.1097/00004703-200604002-00012
17. Hunter JE, Allen EG, Abramowitz A, Rusin M, Leslie M, Novak G, et al. No evidence for a difference in neuropsychological profile among carriers and noncarriers of the FMR1 premutation in adults under the age of 50. *Am J Hum Genet.* (2008) 83:692–702. doi: 10.1016/j.ajhg.2008.10.021
18. Chonchaiya W, Au J, Schneider A, Hessl D, Harris SW, Laird M, et al. Increased prevalence of seizures in boys who were probands with the FMR1 premutation and co-morbid autism spectrum disorder. *Hum Genet.* (2012) 131:581–9. doi: 10.1007/s00439-011-1106-6
19. Brown SSG, Basu S, Whalley HC, Kind PC, Stanfield AC. Age-related functional brain changes in FMR1 premutation carriers. *Neuroimage Clin.* (2017) 17:761–7. doi: 10.1016/j.nicl.2017.12.016
20. Hagerman R, Hagerman P. Advances in clinical and molecular understanding of the FMR1 premutation and fragile X-associated tremor/ataxia syndrome. *Lancet Neurol.* (2013) 12:786–98. doi: 10.1016/S1474-4422(13)70125-X
21. Inoue SB, Siomi MC, Siomi H. Molecular mechanisms of fragile X syndrome. *J Med Invest.* (2000) 47:101–7.
22. Greco CM, Hagerman RJ, Tassone F, Chudley AE, Del Bigio MR, Jacquemont S, et al. Neuronal intranuclear inclusions in a new cerebellar tremor/ataxia syndrome among fragile X carriers. *Brain.* (2002) 125:1760–71. doi: 10.1093/brain/awf184
23. Greco CM, Berman RF, Martin RM, Tassone F, Schwartz PH, Chang A, et al. Neuropathology of fragile X-associated tremor/ataxia syndrome (FXTAS). *Brain.* (2006) 129:243–55. doi: 10.1093/brain/awh683
24. Darnell JC, Van Driesche SJ, Zhang C, Hung KY, Mele A, Fraser CE, et al. FMRP stalls ribosomal translocation on mRNAs linked to synaptic function and autism. *Cell.* (2011) 146:247–61. doi: 10.1016/j.cell.2011.06.013
25. Howard DM, Adams MJ, Shirali M, Clarke TK, Marioni RE, Davies G, et al. Genome-wide association study of depression phenotypes in UK Biobank identifies variants in excitatory synaptic pathways. *Nature Commun.* (2018) 9:1470. doi: 10.1038/s41467-018-03819-3
26. Qin M, Entezam A, Usdin K, Huang T, Liu ZH, Hoffman GE, et al. A mouse model of the fragile X premutation: effects on behavior, dendrite morphology, and regional rates of cerebral protein synthesis. *Neurobiol Dis.* (2011) 42:85–98. doi: 10.1016/j.nbd.2011.01.008
27. Fatemi SH, Folsom TD. The role of fragile X mental retardation protein in major mental disorders. *Neuropharmacology.* (2011) 60:1221–26. doi: 10.1016/j.neuropharm.2010.11.011
28. Hessl D, Tassone F, Loesch DZ, Berry-Kravis E, Leehey MA, Gane LW, et al. Abnormal elevation of FMR1 mRNA is associated with psychological symptoms in individuals with the fragile X premutation. *Am J Med Genet B Neuropsychiatr Genet.* (2005) 139B:115–21. doi: 10.1002/ajmg.b.30241
29. Moore CJ, Daly EM, Tassone F, Tysoe C, Schmitz N, Ng V, et al. The effect of pre-mutation of X chromosome CGG trinucleotide repeats on brain anatomy. *Brain.* (2004) 127:2672–81. doi: 10.1093/brain/awh256
30. Adams JS, Adams PE, Nguyen D, Brunberg JA, Tassone F, Zhang W, et al. Volumetric brain changes in females with fragile X-associated tremor/ataxia syndrome (FXTAS). *Neurology.* (2007) 69:851–9. doi: 10.1212/01.wnl.0000269781.10417.7b
31. Wang JY, Hessl DH, Hagerman RJ, Tassone F, Rivera SM. Age-dependent structural connectivity effects in fragile X premutation. *Arch Neurol.* (2012) 69:482–9. doi: 10.1001/archneurol.2011.2023
32. Hajek T, Kopecek M, Kozeny J, Gunde E, Alda M, Höschl C. Amygdala volumes in mood disorders–meta-analysis of magnetic resonance volumetry studies. *J Affect Disord.* (2009) 115:395–410. doi: 10.1016/j.jad.2008.10.007
33. Spampinato MV, Wood JN, De Simone V, Grafman J. Neural correlates of anxiety in healthy volunteers: a voxel-based morphometry study. *J Neuropsychiatry Clin Neurosci.* (2009) 21:199–205. doi: 10.1176/jnp.2009.21.2.199
34. Iidaka T, Matsumoto A, Ozaki N, Suzuki T, Iwata N, Yamamoto Y, et al. Volume of left amygdala subregion predicted temperamental trait of harm avoidance in female young subjects. A voxel-based morphometry study. *Brain Res.* (2006) 1125:85–93. doi: 10.1016/j.brainres.2006.09.015
35. Hu Y, Moore M, Bertels Z, Phan KL, Dolcos F, Dolcos S. Smaller amygdala volume and increased neuroticism predict anxiety symptoms in healthy subjects: a volumetric approach using manual tracing. *Neuropsychologia.* (2020) 145:106564. doi: 10.1016/j.neuropsychologia.2017.11.008
36. Barrós-Loscertales A, Meseguer V, Sanjuán A, Belloch V, Parcet MA, Torrubia R, et al. Behavioral inhibition system activity is associated with increased amygdala and hippocampal gray matter volume: a voxel-based morphometry study. *Neuroimage.* (2006) 33:1011–15. doi: 10.1016/j.neuroimage.2006.07.025
37. Baur V, Hänggi J, Jäncke L. Volumetric associations between uncinate fasciculus, amygdala, trait anxiety. *BMC Neurosci.* (2012) 13:4. doi: 10.1186/1471-2202-13-4
38. Potvin O, Catheline G, Bernard C, Meillon C, Bergua V, Allard M, et al. Gray matter characteristics associated with trait anxiety in older adults are moderated by depression. *Int Psychogeriatr.* (2015) 27:1813–24. doi: 10.1017/S1041610215000836
39. Blackmon K, Barr WB, Carlson C, Devinsky O, DuBois J, Pogash D, et al. Structural evidence for involvement of a left amygdala-orbitofrontal network in subclinical anxiety. *Psychiatry Res.* (2011) 194:296–303. doi: 10.1016/j.pscychresns.2011.05.007
40. Taki Y, Kinomura S, Awata S, Inoue K, Sato K, Ito H, et al. Male elderly subthreshold depression patients have smaller volume of medial part of prefrontal cortex and precentral gyrus compared with age-matched normal subjects: a voxel-based morphometry. *J Affect Disord.* (2005) 88:313–20. doi: 10.1016/j.jad.2005.08.003
41. Spalletta G, Piras F, Caltagirone C, Fagioli S. Hippocampal multimodal structural changes and subclinical depression in healthy individuals. *J Affect Disord.* (2014) 152-154:105–12. doi: 10.1016/j.jad.2013.05.068
42. Dotson VM, Davatzikos C, Kraut MA, Resnick SM. Depressive symptoms and brain volumes in older adults: a longitudinal magnetic resonance imaging study. *J Psychiatry Neurosci.* (2009) 34:367–75.
43. O'Shea DM, Dotson VM, Woods AJ, Porges EC, Williamson JB, O'Shea A, et al. Depressive symptom dimensions and their association with hippocampal and entorhinal cortex volumes in community dwelling older adults. *Front Aging Neurosci.* (2018) 10:40. doi: 10.3389/fnagi.2018.00040
44. Osler M, Sørensen L, Rozing M, Calvo OP, Nielsen M, Rostrup E. Subclinical depressive symptoms during late midlife and structural brain alterations: a longitudinal study of Danish men born in 1953. *Hum Brain Mapp.* (2018) 39:1789–95. doi: 10.1002/hbm.23954
45. Goveas JS, Espeland MA, Hogan P, Dotson V, Tarima S, Coker LH, et al. Depressive symptoms, brain volumes and subclinical cerebrovascular

disease in postmenopausal women: the Women's Health Initiative MRI Study. *J Affect Disord.* (2011) 132:275–84. doi: 10.1016/j.jad.2011.01.020
46. Szymkowicz SM, Woods AJ, Dotson VM, Porges EC, Nissim NR, O'Shea A, et al. Associations between subclinical depressive symptoms and reduced brain volume in middle-aged to older adults. *Aging Mental Health.* (2019) 23:819–30. doi: 10.1080/13607863.2018.1432030
47. Abitbol M, Menini C, Delezoide AL, Rhyner T, Vekemans M, Mallet J. Nucleus basalis magnocellularis and hippocampus are the major sites of FMR-1 expression in the human fetal brain. *Nature Genet.* (1993) 4:147–53. doi: 10.1038/ng0693-147
48. Tassone F, Hagerman RJ, Garcia-Arocena D, Khandjian EW, Greco CM, Hagerman PJ. Intranuclear inclusions in neural cells with premutation alleles in fragile X associated tremor/ataxia syndrome. *J Med Genet.* (2004) 41:e43. doi: 10.1136/jmg.2003.012518
49. Tassone F, Iwahashi C, Hagerman PJ. FMR1 RNA within the intranuclear inclusions of fragile X-associated tremor/ataxia syndrome (FXTAS). *RNA Biol.* (2004) 1:103–5. doi: 10.4161/rna.1.2.1035
50. Entezam A, Biacsi R, Orrison B, Saha T, Hoffman GE, Grabczyk E, et al. Regional FMRP deficits and large repeat expansions into the full mutation range in a new Fragile X premutation mouse model. *Gene.* (2007) 395:125–34. doi: 10.1016/j.gene.2007.02.026
51. Johnson K, Herring J, Richstein J. Fragile X Premutation Associated Conditions (FXPAC). *Front Pediatr.* (2020) 8:266. doi: 10.3389/fped.2020.00266
52. Clifton, N. E., Rees, E., Holmans, P. A. et al. Genetic association of FMRP targets with psychiatric disorders. *Mol Psychiatry* (2020). doi: 10.1038/s41380-020-00912-2

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The Use of Peptides in the Treatment of Fragile X Syndrome: Challenges and Opportunities

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Fragile X Syndrome (FXS) is the most frequent cause of inherited intellectual disabilities and autism spectrum disorders, characterized by cognitive deficits and autistic behaviors. The silencing of the *Fmr1* gene and consequent lack of FMRP protein, is the major contribution to FXS pathophysiology. FMRP is an RNA binding protein involved in the maturation and plasticity of synapses and its absence culminates in a range of morphological, synaptic and behavioral phenotypes. Currently, there are no approved medications for the treatment of FXS, with the approaches under study being fairly specific and unsatisfying in human trials. Here we propose peptides/peptidomimetics as candidates in the pharmacotherapy of FXS; in the last years this class of molecules has catalyzed the attention of pharmaceutical research, being highly selective and well-tolerated. Thanks to their ability to target protein-protein interactions (PPIs), they are already being tested for a wide range of diseases, including cancer, diabetes, inflammation, Alzheimer's disease, but this approach has never been applied to FXS. As FXS is at the forefront of efforts to develop new drugs and approaches, we discuss opportunities, challenges and potential issues of peptides/peptidomimetics in FXS drug design and development.

Keywords: Fragile X syndrome, targeted therapy, peptides, peptidomimetics, drug development

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INTRODUCTION

Fragile X syndrome (FXS) was first described in 1943 and it is now established as the most common cause of inheritable intellectual disabilities (ID) (1). The molecular cause of FXS is the extensive repeat expansion of a CGG triplet (200 repeats in the full mutation) in the 5′ untranslated region (UTR) and consequential hypermethylation of the *Fmr1* gene, finally leading to transcriptional silencing of the fragile X mental retardation protein (FMRP) (2–4). A small proportion of individuals affected by FXS with different levels of severity show deletion or point mutation in the *Fmr1* gene, that in turn cause the complete loss of FMRP or the production of a functionally deficient protein (5–7).

This disease affects 1:4,000 males and 1:6,000–8,000 females (8, 9) showing symptoms from moderate to severe ID (10). The clinical picture of the syndrome is complex; FXS phenotype displays characteristics in common with autism spectrum disorder (ASD) and attention deficit hyperactivity disorder (ADHD), with general anxiety, social avoidance and hyperactive behaviors (11–13). Seizures, recurrent otitis media, strabismus and obesity are also often occurring in patients affected by FXS. Besides, about 10% of males with FXS display a Prader-Willi like phenotype (14).

The physical features of FXS comprehend elongated face, broad forehead, high palate, prominent ears, hyperextensible finger joints, flat feet and macroorchidism (15). All these behavioral, phenotypical and clinical characteristics of FXS, are due to the lack of FMRP, a well-characterized RNA-binding protein, showing crucial functions mainly related to mRNAs metabolism (16). Its main role is represented by the translational repression of numerous key mRNAs in pre- and postsynaptic neurons (17, 18). The FMRP deficiency results in increased protein synthesis, causing the upregulation of several signaling effectors, such as excitatory metabotropic glutamate receptor (mGluR) (19, 20), α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) receptor (21), extracellular signal-related kinase (ERK1/2) (22–24), matrix metalloproteinase 9 (MMP-9) (25–27), brain-derived neurotrophic factor (BDNF) (28, 29), and mammalian target of rapamycin (mTOR) (30, 31). Moreover, functional impairment in gamma-aminobutyric acid (GABA) receptor and in the endocannabinoid system have been also documented in FXS (32–34). In healthy conditions all these machineries orchestrate neurotransmission and local protein synthesis that impact synaptic plasticity, learning and memory. Hence, in FXS pathology, the lack of FMRP leads to increased protein synthesis with a direct effect on dendritic spine dysgenesis and cognitive disabilities (9, 17), causing the majority of the FXS symptoms. Evidences from *Fmr1* knockout (KO) mice and from human post-mortem brain biopsies showed increased amount and length of dendritic spines, with an immature profile (35–37).

FMRP inhibits translation initiation through its interactions with eIF4E (Eukaryotic translation Initiation Factor 4E) and CYFIP1 (Cytoplasmic FMRP Interacting Protein 1) (4, 7, 38–40). eIF4E is the cap-binding protein known to be activated by the interaction with the scaffold protein eIF4G (Eukaryotic translation Initiation Factor 4G) or inhibited by 4E-binding proteins (4E-BPs), these last being a well-characterized group of proteins that repress protein synthesis (41, 42). The 4E-BPs and eIF4G compete for the same binding site on the eIF4E surface; thus, 4E-BPs inhibit the eIF4E-eIF4G complex formation by sequestering the unbound eIF4E (43, 44). CYFIP1 belongs to the 4E-BPs family and in neurons, mainly at synapses, the FMRP-CYFIP1-eIF4E inhibitory complex regulates protein synthesis during synaptic activity, playing a pivotal role in the modulation of long-term synaptic plasticity at synapses (18, 38). Moreover, the CYFIP1 paralog CYFIP2 is itself able to interact with FMRP and with the FMRP-related proteins FXR1P/2P, which are cytoplasmatic proteins that share with FMRP the functional domains deputed to promote homo- and heteromerization (45, 46).

CURRENT STRATEGIES IN FXS TREATMENT

Recently, strong effort was dedicated to develop specific FXS pharmacological treatment that can lead to a possible cure, or at least alleviate symptoms (47–51). The most promising or studied treatments for FXS are listed in **Table 1**. However, although several therapeutic approaches are being tested on different FXS animal models (i.e., *Fmr1* KO mouse, rat and zebrafish; *dFmr* null mutant fly) and patients over the years, an approved and successful curative therapy for FXS is missing to date, and the management of the clinical aspects of the syndrome continue to focus on symptomatic treatment of psychiatric and behavioral problems, rather than the molecular causes (49, 50, 52).

One of the first approaches that was suggested for FXS treatment was the *Fmr1* gene activity restoration through changes in the DNA methylation levels and epigenetic modifications (53, 54). Although different compounds were tested and successfully achieving *in vitro* reactivation of the *Fmr1* gene, such as 5-azadeoxycytidine (5-azadC) (53), this strategy has not been tested with *in vivo* studies due to safety problems related to low reactivity and high toxicity of these chromatin-modifying enzymes inhibitors (55). Similarly, another strategy involves the use of non-coding RNAs to affect DNA methylation state and histones modification (56). Based on the promising results obtained in cancer and other diseases (57), several miRNAs and lncRNAs were identified and tested in different FXS models (58–60), but their potential use in clinical therapy is still far away similarly to the modern application of gene therapy methods to restore the *Fmr1* gene (61). Indeed, independent groups demonstrated the possibility to use viral-vectors or CRISPR-technology, with encouraging results in preclinical FXS models (62–67); however, the clinical application in patients is being debated for several undisclosed questions, as safety and brain-targeted delivery. Regarding this approach it is also important to consider that the reactivation of the *Fmr1* mRNA with the full mutation could be toxic, as it was demonstrated by a correlation detected between the *Fmr1* mRNA levels in blood and more severe autism features (68).

However, since several compounds are used to treat behavioral and mental problems, such as stimulants or antipsychotics (69), the majority of pharmacological efforts are employed to compensate the absence of FMRP. Among targeted treatment for FXS, several focused on the neurotransmission imbalance associated with FXS. Particular attention has been dedicated in testing the group 1 metabotropic glutamate receptors 5 (mGluR5) antagonists, such as AFQ056/Mavoglutarant, Fenobam, MPEP, STX107, CTEP, RO4917523 (9, 10, 17, 19), and GABA receptors (GABAa and GABAb) agonists (70). In *Fmr1* KO mice these agents showed improvement of several FXS features, including better behavioral abilities, restoration of normal levels of dendritic spines and reduction in protein synthesis (9, 17, 69). Despite these positive results, the transition from animal to human model did not give the same encouraging outcomes, since most of clinical trials failed (71, 72). The high placebo response and the imprecise design and methodology of the trials were the major causes of failure.

Most of drugs tested in FXS pharmacotherapy are compounds already employed or approved for other disorders. Sertraline is a serotonin reuptake inhibitor approved for treating anxiety and mental disabilities in young children and tested in *Fmr1*-KO mouse model. Sertraline normalizes serotonin and dopamine levels, with a rescue on synapse and dendritic formation (73, 74). Even in FXS patients, Sertraline showed favorable results, as several studies demonstrated improvements

in language, anxiety and social conduct (75, 76). Cannabidiol (CBD), a synthetic molecule active on cannabinoid receptors, has been used for the treatment of neurological disorders, such as Huntington, Parkinson's and Alzheimer's diseases, but also epilepsy, schizophrenia, autoimmune diseases. All these pathologies have in common altered endocannabinoid signaling pathway, condition confirmed to be deregulated also in FXS animal model (77, 78). Clinical studies indicated good results (79–81), albeit with tolerated side effects. The following FDA-approved drugs have been tested in FXS preclinical and clinical studied: acamprosate (for maintenance of alcohol abstinence), lovastatin (for hypercholesterolemia), minocycline (for acne) and metformin (for non-insulin diabetes mellitus). In particular lovastatin targets the RAS-MAPK-ERK1/2 pathway (82, 83) while minocycline inhibits the MMP-9 activity (25). Both compounds showed promising results in preclinic testing using different model systems, with a reduction in protein synthesis and beneficial cognitive and behavioral aspects (25, 82, 84, 85). Nevertheless, these encouraging data were followed by moderate effects in trials on FXS patients, also expressing the need for a more in-depth investigation on the tolerability of these compounds (86–88). To date, the anti-diabetes drug metformin could be considered as one of the most promising treatments for FXS (89, 90). It has different mechanisms of action, depending on dosage and treatment time, including inhibition of mammalian/mechanistic target of rapamycin complex 1 (mTORC1) and mitogen-activated protein kinase/extracellular signal regulated kinase (MAPK/ERK) pathways, both hyperactivated due to the lack of FMRP in FXS humans and mice (91, 92). As a consequence, metformin also affects the proteins downstream to these cascades, reducing specifically the eIF4E phosphorylation and the translation of MMP-9 (93), which in the pathological condition is the cause for the degradation of proteins essential for synaptic maturation and activity (27). Preclinical studies were performed on *Fmr1*-KO flies and mice models of FXS, showing a rescue of dendritic spine morphology, long-term depression (LTD) of synapses, but also improvement in cognitive, intellectual and social deficits (90, 94). These findings paved the way for treatments in humans, where clinical trials starting in 2018 have been conducted with promising benefits both in terms of behavior and safety of treatment (93, 95–97). Currently 3 trials aimed to evaluate safety, tolerability, and efficacy of metformin in FXS patients are ongoing (ClinicalTrials.gov Identifier: NCT04141163, NCT03862950, NCT03479476). Another interesting therapeutic target in FXS is represented by phosphodiesterases (PDEs), a family of enzymes that regulate the cellular levels of cAMP and cGMP. Among PDEs, PDE1A, PDE2A, and PDE10A have been identified as mRNA targets of FMRP (98). Accordingly, decreased cAMP levels were observed in fly and mouse FXS models and a deregulation of cAMP and cGMP was also identified as a molecular hallmark in FXS patients (99, 100). Hence, several inhibitors have been tested, starting from *Drosophila* model of fragile X, passing through *Fmr1-KO* mice, finally to human trials. Interestingly, inhibiting PDE4 (101, 102), PDE2A (103, 104), PDE4D (105, 106), or synergistically PDE2 and PDE4 (107) demonstrated beneficial effects in terms of rescue of social and behavioral impairments and in dendritic spines morphology in fly and mouse models. Cognitive enhancements were pointed out from FXS trials (105, 106), suggesting that PDEs are candidate targets to develop FXS therapeutic strategy.

PEPTIDES/PEPTIDOMIMETICS: A FEASIBLE STRATEGY FOR FXS TREATMENT

All the strategies mentioned so far target different pathways, whose uncontrolled activity seems to be crucial in the pathology of FXS, but also in other neurological disorders and types of cancer (108, 109), leading to pleiotropic effects. Accordingly, the lack of specificity and selectivity, together with bioavailability and safety problems, could be the main drawbacks of these approaches.

In this scenario, a novel and feasible option in FXS pharmacotherapy could be the use of peptides or peptidomimetics.

Since the last 30 years, and especially in the past decade, severe pathologies are being treated with peptides (110) and this class of molecules have attracted the attention of either academia researchers or pharmaceutical industries. Indeed, the global Peptide Therapeutics market reached USD 25.35 billion in 2018 and is expected to achieve USD 50.60 billion by the year 2026. To date, 400–600 peptides are in the preclinical phase of development and more than 60 peptides are FDA-approved (111). The main fields in which therapeutic peptides are currently in development are oncology, metabolic diseases and inflammation (110). Peptides represent an attractive pharmaceutical source due to their excellent properties, namely high selectivity, safety and tolerability (112). However, this kind of approach has never been applied to FXS to restore the imbalance in protein synthesis. Very recently novel structural information opened new possibilities for developing inhibitors acting on the mRNAs translation initiation complex with high specificity and efficiency.

Among these compounds, the 4EGI-1 is one of the most promising inhibitors of the translation activation complex and it has been already tested in different cancer models (113–116). This molecule was also proved to reduce the eIF4E-eIF4G interaction in a FXS mouse model (117). However, the lack of drug-like characteristics, such as poor target specificity and selectivity, high toxicity, several off-targets, severe side effects, poor metabolic stability, poor membrane permeability and rapid proteolysis, make this molecule fairly unsuitable candidates for therapeutic applications.

Furthermore, the putative molecules effective in disrupting the FMRP-CYFIP1-eIF4E or eIF4E-eIF4G complexes formation, are required to target protein-protein interaction (PPI) interfaces, that are large, flat and hydrophobic binding surfaces considered as "undruggable" by small compounds (118–120). One solution could be represented by antibodies, more powerful in targeting PPI, but anyway scarcely able to cross the cell membrane to perform their specific function. In light of this, peptides are

TABLE 1 | Treatments for Fragile X syndrome.

Approach	Name	Agent class	Mechanism of action
Fmr1 gene activity restoration	5-azadeoxycytidine (5-azadC)	Chromatin-modifying enzymes inhibitor	Affects DNA methylation levels and epigenetic modifications
		Non-coding RNA (miRNAs and lncRNAs)	Affect DNA methylation state and histones modification
		Viral-vectors or CRISPR-technology	Gene editing or gene replacement
Targeted therapy	AFQ056/Mavoglutarant, Fenobam, MPEP, STX107, CTEP, RO4917523	Group 1 metabotropic glutamate receptors 5 (mGluR5) antagonists	Block mGluRI signaling
	OV101/gaboxadol, Ganaxolone	GABAa and GABAb agonists	Modulate GABA receptors
	Sertraline	Serotonin reuptake inhibitor	Normalizes serotonin and dopamine levels, stimulation of BDNF
	Cannabidiol	Cannabinoid receptors inhibitor	Regulates endocannabinoid signaling pathway
	Lovastatin	RAS signaling inhibitor	Regulates RAS-MAPK-ERK1/2 pathway
	Minocycline	Semi-synthetic tetracycline derivative	Regulates MMP-9 activity
	Metformin	Derivative of guanidine	Normalizes mTOR and MAPK/ERK pathways, phosphorylates eIF4E, and lowers expression of MMP9
	Bay 60–7550, BNP14770, Roflumilast	PDE4, PDE2A, PDE4D inhibitors	Normalize cAMP and cGMP signaling
Symptomatic treatment	Risperidone and aripiprazole	Antipsychotics	Impacts dopaminergic and serotonergic neurotransmission to treat irritability

now considered as the most appropriate candidates to regulate disease-associated PPIs. However, peptides have intrinsic weak points, and they did not provide encouraging results *in vivo*, likely due to their physical, chemical and structural instability and low membrane permeability (112, 119, 121). To overcome these possible limitations several strategies have been developed, such as amino acids substitution with residues mimicry, termini protection or introduction of chemical modifications aimed at stabilizing their active conformation and increasing cellular permeability (119, 121). These advances in the peptides technology results in the development of an alternative class of compounds called peptidomimetics, that is recently emerging as a class of new potential therapeutic molecules able to target PPIs in the treatment of different pathologies (112). Peptidomimetics are organic molecules with physico-chemical features and structural characteristics comparable with classical oligopeptides, but guarantee enhanced protection against peptidases, improved systemic delivery and cellular uptake, high target specificity and poor immune response (122), and for these reasons their use is under investigation for the treatment of cancer, ischemia, Alzheimer's disease (123–127) and other neurodegenerative disorders (128–131).

On the contrary, the use of peptides/peptidomimetics has never been investigated in the FXS context, but it could represent a viable solution as it might result in a compensation of FMRP absence. Indeed, restoring the FMRP-CYFIP1 deficiency *via* a small chimeric peptide acting on the dysregulation of protein synthesis could be central for the new FXS pharmacological therapy development.

Although the 3D structure of FMRP-CYFIP1 or FMRP with other interacting proteins are still not available, there is a growing number of structures, from different organisms, of complexes belonging to the translation initiation pathway, in particular eIF4G/eIF4E and eIF4E/4E-BPs, and of their regulatory proteins (43, 44, 132, 133). The plethora of structural information, together with the increasing power of computational facilities and refinement of binding prediction tools (110, 112, 119), open the possibility to design peptides/peptidomimetics able to target the translation complexes, with the aim to decrease protein synthesis of specific neuronal mRNAs and rescuing a healthy phenotype in individuals affected by FXS.

For instance, peptides could target and affect the assembly of the eukaryotic Initiation Factor 4F (eIF4F) complex (composed by eIF4E, the DEAD-box helicase eIF4A and the scaffold protein eIF4G) (41, 42), and the formation of 43S pre-initiation complex (43S PIC), composed by small ribosomal subunit 40S and the eukaryotic translation initiation factors (eIFs): eIF1, eIF1A, eIF3, eIF5 (41, 42) (**Figure 1**). In addition to those already mentioned, the PPIs that could be targeted and disrupted by peptides, could be for instance, eIF4E/cap, eIF4A/eIF4G or eIF4G/eIF3 dimers (**Figure 1**). Alternatively, peptides/peptidomimetics could be designed to target the eIF4E-upstream regulators, as the PI3K–mTOR pathway, likely inhibiting the interactions of mTORC1 with Raptor and other partners, affecting the activity of the downstream S6K or 4E-BP proteins (**Figure 1**) (134). Furthermore, not only protein synthesis but also actin dynamic imbalance concurs to the pathophysiology of FXS, leading to defects in dendritic spines morphology (135, 136). Although much information is still lacking, there are indications that Rac1–PAK pathway and FMRP are linked (137, 138). Active Rac1, the Rho-family of small GTPases, activates p21-activated kinases (PAKs) which in turn phosphorylates Cofilin, an actin-binding protein that regulates actin turnover. Rac1 also activates the Wave Regulatory Complex (WRC, composed by five proteins:

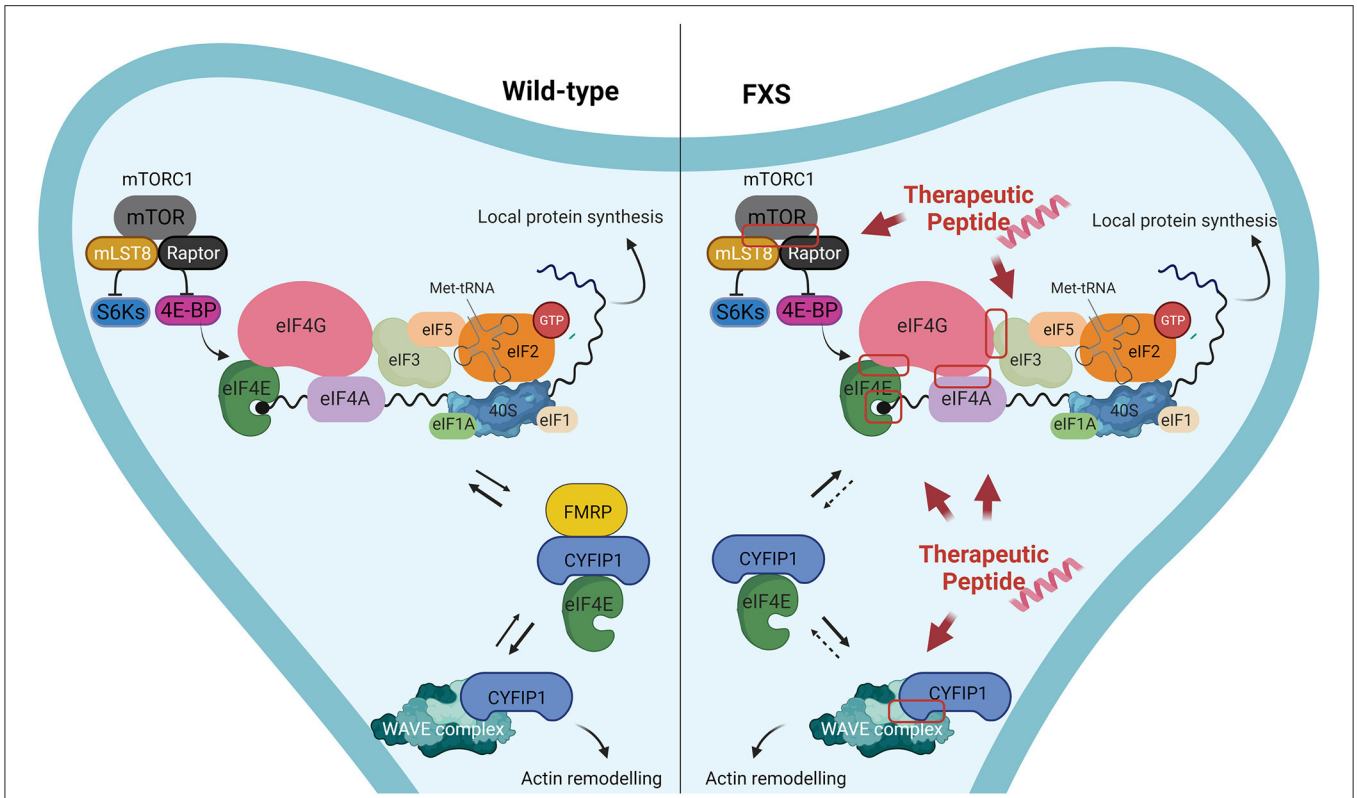

FIGURE 1 | Proposed examples of protein-protein interactions that could be targeted by peptides/peptidomimetics in Fragile X Syndrome. Left panel: In wild-type neurons FMRP plays a key role in down-regulating the translation of FMRP targets, by forming a complex with CYFIP1 and the eukaryotic initiation factor 4E (eIF4E). Furthermore, CYFIP1 can bind and inhibit the WAVE regulatory complex, thereby regulating actin remodeling. Right panel: In FXS neurons the absence of FMRP leads to increased local protein synthesis in neurons, due to the lack of the formation CYFIP1-FMRP-eIF4E complex, that represses the translation initiation. Consequently, a bigger amount of eIF4E is bound to eIF4G, while CYFIP1 interacts mostly with WRC-Rac1-GTP, resulting in higher levels of protein synthesis an altered actin remodeling at dendritic spines. Examples of macromolecular complexes that could be disrupted by peptides/peptidomimetics with the aim to restore the FMRP activity are highlighted by a red box.

CYFIP1, NCKAP1, Abi2, HSPC300, and WAVE1) by directly binding CYFIP1 and leading to Arp2/3 complex-mediated actin polymerization (139–141). The release of the X-ray structure of WRC (140), together with data coming from computational analyses that highlighted structural-dynamical features of CYFIP1 (39, 40), may provide useful details to be exploited for the CYFIP1-based peptidomimetics design (**Figure 1**). To offer some realistic examples, using different regions of CYFIP1 as templates, CYFIP1-derived peptidomimetics could interfere with the eIF4F complex formation by sequestering eIF4E from the binding with eIF4G. Similarly, impeding the CYFIP1/Rac1 interaction or the CYFIP1/NCKAP1 dimer formation, could have a dual beneficial effect in concomitantly restoring normal levels of protein synthesis and actin dynamics, both processes being dysregulated in FXS. Aside from CYFIP1, other 4E-BPs structures in complex with eIF4E are available and represent an attractive template for peptides design. For example, 4E-BP1, 4E-BP2, and Angel1-based peptides were developed and tested in different cancer cell lines (142–144). Furthermore, Lama et al. developed a set of peptides with chemical modifications that increase the pharmacological properties and binding affinity to eIF4E, providing a strong starting point for future oncological preclinical studies (145–147).

On the basis of the knowledge acquired in cancer research, we assume that the peptides/peptidomimetics approach could also be applied to other diseases, in particular in the FXS pharmacotherapeutic.

DISCUSSION

In the last decade many efforts were employed in research and development of new pharmacological treatments of FXS and simultaneously great advances were made in developing peptides therapeutics against several diseases. With this perspective we speculate that these two roads might cross, starting a new era of the pharmacotherapeutic approach for patients with FXS. To date, chemical compounds that inhibit several pathways deregulated in FXS represent the most studied approaches for FXS management. However, the cause of FXS is a genetic defect (the *Fmr1* silencing) and to effectively correct the absence of FMRP protein is still a challenge. Moreover, although several

available treatments are effective in animal models, many clinical trials testified lack of success of these approaches. The emerging peptides technology, in combination with increasingly advanced computational approaches and number of proteins structures deposited in databases, provide an alternative and powerful method to develop a more specific and safe molecules targeting those protein complexes that could be considered as the major players in the FXS pathology. The main point of strength of the use of peptides include selectivity, tolerability, predictable metabolism, ability to target PPIs and lower synthesis complexity that in turn leads to lower production costs compared to others pharmaceutical molecules (119, 121). However, as mentioned above, peptides in general, and thus their possible application against FXS, have several weaknesses that is necessary to discuss. Poor *in vivo* stability, membrane impermeability, and toxicity are widely accounted as major drawbacks in peptides technology. Nevertheless, several of these aspects have been successfully sorted out over recent years through the new technologies available in peptides design field: new bioinformatics tools in combination with other approaches such as virtual screening, structure-based drug design, high throughput screening (HTS) and chemical strategies, provide a comprehensive pharmacological description of putative peptides, improving their chemical and physical features (119, 121, 148). Moreover, the treatment of neurodevelopmental disorders, as FXS, require the delivery of molecules to the brain, accounting for the crossing of the blood–brain barrier (BBB), that could limit the access of peptides to the central nervous system (CNS). In this respect, a strategy to overcome these issues could be the exploration of other drugs administration methods, e.g., the non-invasive intranasal delivery (129, 131). The direct access to CNS allows to overcome limitations linked to the degradation, bioavailability problems and also to possible systemic side effects onset that occur if peptides are present in blood vessels after intravenous administration. Additionally, several strategies have been developed to specifically deliver peptides to target regions of the CNS, such as cyclodextrins, PEI or others (129), resulting in a lower dosage of peptides, also decreasing the toxicity issues which have been demonstrated toward eukaryotic cells (149, 150).

Furthermore, peptides therapy would not be a chronic intervention, but these molecules would be administered only in a limited period of time during the 1st years of life of FXS children, when brain is still remodeling, to allow proper formation of the synaptic network.

Hence, we propose that peptides/peptidomimetics could compensate the FMRP deficiency restoring the imbalance of protein synthesis and actin dynamics, suggesting a new and promising strategy for treating FXS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

This perspective was conceived by DD and AR. DD and AR wrote the manuscript. Table and figure were prepared by AR. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Martin JP, Bell J. A pedigree of mental defect showing sex-linkage. *J Neurol Psychiatry.* (1943) 6:154–7. doi: 10.1136/jnnp.6.3-4.154
2. Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP, Pizzuti A, et al. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell.* (1991) 65:905–14. doi: 10.1016/0092-8674(91)90397-H
3. Oberlé I, Rousseau F, Heitz D, Kretz C, Devys D, Hanauer A, et al. Instability of a 550-base pair DNA segment and abnormal methylation in fragile X syndrome. *Science.* (1991) 252:1097–102. doi: 10.1126/science.252.5009.1097
4. D'Annessa I, Cicconardi F, Di Marino D. Handling FMRP and its molecular partners: structural insights into Fragile X Syndrome. *Prog Biophys Mol Biol.* (2019) 141:3–14. doi: 10.1016/j.pbiomolbio.2018.07.001
5. Myrick LK, Nakamoto-Kinoshita M, Lindor NM, Kirmani S, Cheng X, Warren ST. Fragile X syndrome due to a missense mutation. *Eur J Hum Genet.* (2014) 22:1185–9. doi: 10.1038/ejhg.2013.311
6. Quartier A, Poquet H, Gilbert-Dussardier B, Rossi M, Casteleyn AS, Portes V des, et al. Intragenic FMR1 disease-causing variants: a significant mutational mechanism leading to Fragile-X syndrome. *Eur J Hum Genet.* (2017) 25:423–31. doi: 10.1038/ejhg.2016.204
7. Di Marino D, Achsel T, Lacoux C, Falconi M, Bagni C. Molecular dynamics simulations show how the FMRP Ile304Asn mutation destabilizes the KH2 domain structure and affects its function. *J Biomol Struct Dyn.* (2014) 32:337–50. doi: 10.1080/07391102.2013.768552
8. Pugin A, Faundes V, Santa María L, Curotto B, Aliaga S, Salas I, et al. Clinical, molecular, and pharmacological aspects of FMR1-related disorders. *Neurología.* (2017) 32:241–52. doi: 10.1016/j.nrleng.2014.10.018
9. Hagerman RJ, Berry-Kravis E, Hazlett HC, Bailey DBJ, Moine H, Kooy RF, et al. Fragile X syndrome. *Nat Rev Dis Prim.* (2017) 3:17065. doi: 10.1038/nrdp.2017.65
10. Richter JD, Bassell GJ, Klann E. Dysregulation and restoration of translational homeostasis in fragile X syndrome. *Nat Rev Neurosci.* (2015) 16:595–605. doi: 10.1038/nrn4001
11. Thurman AJ, McDuffie A, Hagerman R, Abbeduto L. Psychiatric symptoms in boys with fragile X syndrome: a comparison with nonsyndromic autism spectrum disorder. *Res Dev Disabil.* (2014) 35:1072–86. doi: 10.1016/j.ridd.2014.01.032
12. McDuffie A, Thurman AJ, Hagerman RJ, Abbeduto L. Symptoms of autism in males with fragile X syndrome: a comparison to nonsyndromic ASD using current ADI-R scores. *J Autism Dev Disord.* (2015) 45:1925–37. doi: 10.1007/s10803-013-2013-6
13. Berry-Kravis E, Raspa M, Loggin-Hester L, Bishop E, Holiday D, Bailey DB. Seizures in fragile X syndrome: characteristics and comorbid diagnoses. *Am J Intellect Dev Disabil.* (2010) 115:461–72. doi: 10.1352/1944-7558-115.6.461
14. Nowicki ST, Tassone F, Ono MY, Ferranti J, Croquette MF, Goodlin-Jones B, et al. The Prader-Willi phenotype of fragile X syndrome. *J Dev Behav Pediatr.* (2007) 28:133–8. doi: 10.1097/01.DBP.0000267563.18952.c9
15. Salcedo-Arellano MJ, Dufour B, McLennan Y, Martinez-Cerdeno V, Hagerman R. Fragile X syndrome and associated

disorders: clinical aspects and pathology. *Neurobiol Dis.* (2020) 136:104740. doi: 10.1016/j.nbd.2020.104740
16. Richter JD, Zhao X. The molecular biology of FMRP: new insights into fragile X syndrome. *Nat Rev Neurosci.* (2021) 22:209–22. doi: 10.1038/s41583-021-00432-0
17. Bagni C, Neri G, Hagerman R. Fragile X syndrome: causes, diagnosis, mechanisms, and therapeutics. *J Clin Invest.* (2012) 122:4314–22. doi: 10.1172/JCI63141
18. De Rubeis S, Bagni C. Fragile X mental retardation protein control of neuronal mRNA metabolism: insights into mRNA stability. *Mol Cell Neurosci.* (2010) 43:43–50. doi: 10.1016/j.mcn.2009.09.013
19. Bear MF, Huber KM, Warren ST. The mGluR theory of fragile X mental retardation. *Trends Neurosci.* (2004) 27:370–7. doi: 10.1016/j.tins.2004.04.009
20. Gross C, Berry-Kravis EM, Bassell GJ. Therapeutic strategies in fragile X syndrome: dysregulated mGluR signaling and beyond. *Neuropsychopharmacology.* (2012) 37:178–95. doi: 10.1038/npp.2011.137
21. Cheng GR, Li XY, Xiang YD, Liu D, Mcclintock SM, Zeng Y. The implication of AMPA receptor in synaptic plasticity impairment and intellectual disability in fragile X syndrome. *Physiol Res.* (2017) 66:715–27. doi: 10.33549/physiolres.933473
22. Kim SH, Markham JA, Weiler IJ, Greenough WT. Aberrant early-phase ERK inactivation impedes neuronal function in fragile X syndrome. *Proc Natl Acad Sci USA.* (2008) 105:4429–34. doi: 10.1073/pnas.0800257105
23. Matic K, Eninger T, Bardoni B, Davidovic L, Macek B. Quantitative phosphoproteomics of Murine Fmr1 -KO cell lines provides new insights into FMRP-dependent signal transduction mechanisms. *J Proteome Res.* (2014) 13:4388–97. doi: 10.1021/pr5006372
24. Curia G, Gualtieri F, Bartolomeo R, Vezzali R, Biagini G. Resilience to audiogenic seizures is associated with p-ERK1/2 dephosphorylation in the subiculum of Fmr1 knockout mice. *Front Cell Neurosci.* (2013) 7:46. doi: 10.3389/fncel.2013.00046
25. Bilousova TV, Dansie L, Ngo M, Aye J, Charles JR, Ethell DW, et al. Minocycline promotes dendritic spine maturation and improves behavioural performance in the fragile X mouse model. *J Med Genet.* (2009) 46:94–102. doi: 10.1136/jmg.2008.061796
26. Dansie LE, Phommahaxay K, Okusanya AG, Uwadia J, Huang M, Rotschafer SE, et al. Long-lasting effects of minocycline on behavior in young but not adult fragile X mice. *Neuroscience.* (2013) 246:186–98. doi: 10.1016/j.neuroscience.2013.04.058
27. Michaluk P, Wawrzyniak M, Alot P, Szczot M, Wyrembek P, Mercik K, et al. Influence of matrix metalloproteinase MMP-9 on dendritic spine morphology. *J Cell Sci.* (2011) 124:3369–80. doi: 10.1242/jcs.090852
28. Kim SW, Cho KJ. Activity-dependent alterations in the sensitivity to BDNF-TrkB signaling may promote excessive dendritic arborization and spinogenesis in fragile X syndrome in order to compensate for compromised postsynaptic activity. *Med Hypotheses.* (2014) 83:429–35. doi: 10.1016/j.mehy.2014.07.007
29. Castrén M, Lampinen KE, Miettinen R, Koponen E, Sipola I, Bakker CE, et al. BDNF regulates the expression of fragile X mental retardation protein mRNA in the hippocampus. *Neurobiol Dis.* (2002) 11:221–9. doi: 10.1006/nbdi.2002.0544
30. Sharma A, Hoeffer CA, Takayasu Y, Miyawaki T, McBride SM, Klann E, et al. Dysregulation of mTOR signaling in fragile X syndrome. *J Neurosci.* (2010) 30:694–702. doi: 10.1523/JNEUROSCI.3696-09.2010
31. Price TJ, Rashid MH, Millecamps M, Sanoja R, Entrena JM, Cervero F. Decreased nociceptive sensitization in mice lacking the Fragile X mental retardation protein: role of mGluR1/5 and mTOR. *J Neurosci.* (2007) 27:13958–67. doi: 10.1523/JNEUROSCI.4383-07.2007
32. Centonze D, Rossi S, Mercaldo V, Napoli I, Ciotti MT, De Chiara V, et al. Abnormal striatal GABA transmission in the mouse model for the fragile X syndrome. *Biol Psychiatry.* (2008) 63:963–73. doi: 10.1016/j.biopsych.2007.09.008
33. Curia G, Papouin T, Séguéla P, Avoli M. Downregulation of tonic GABAergic inhibition in a mouse model of fragile X syndrome. *Cereb Cortex.* (2009) 19:1515–20. doi: 10.1093/cercor/bhn159
34. D'Hulst C, De Geest N, Reeve SP, Van Dam D, De Deyn PP, Hassan BA, et al. Decreased expression of the GABAA receptor in fragile X syndrome. *Brain Res.* (2006) 1121:238–45. doi: 10.1016/j.brainres.2006.08.115
35. Pan F, Aldridge GM, Greenough WT, Gan WB. Dendritic spine instability and insensitivity to modulation by sensory experience in a mouse model of fragile X syndrome. *Proc Natl Acad Sci USA.* (2010) 107:17768–73. doi: 10.1073/pnas.1012496107
36. Hinton VJ, Brown WT, Wisniewski K, Rudelli RD. Analysis of neocortex in three males with the fragile X syndrome. *Am J Med Genet.* (1991) 41:289–94. doi: 10.1002/ajmg.1320410306
37. Comery TA, Harris JB, Willems PJ, Oostra BA, Irwin SA, Weiler IJ, et al. Abnormal dendritic spines in fragile X knockout mice: maturation and pruning deficits. *Proc Natl Acad Sci USA.* (1997) 94:5401–4. doi: 10.1073/pnas.94.10.5401
38. Napoli I, Mercaldo V, Boyl PP, Eleuteri B, Zalfa F, De Rubeis S, et al. The fragile X syndrome protein represses activity-dependent translation through CYFIP1, a new 4E-BP. *Cell.* (2008) 134:1042–54. doi: 10.1016/j.cell.2008.07.031
39. Di Marino D, Chillemi G, De Rubeis S, Tramontano A, Achsel T, Bagni C. MD and docking studies reveal that the functional switch of CYFIP1 is mediated by a butterfly-like motion. *J Chem Theory Comput.* (2015) 11:3401–10. doi: 10.1021/ct500431h
40. Di Marino D, D'Annessa I, Tancredi H, Bagni C, Gallicchio E. A unique binding mode of the eukaryotic translation initiation factor 4E for guiding the design of novel peptide inhibitors. *Protein Sci.* (2015) 24:1370–82. doi: 10.1002/pro.2708
41. Sonenberg N, Hinnebusch AG. Regulation of translation initiation in eukaryotes. *Cell.* (2009) 136:731–45. doi: 10.1016/j.cell.2009.01.042
42. Merrick WC, Pavitt GD. Protein synthesis initiation in eukaryotic cells. *Cold Spring Harb Perspect Biol.* (2018) 10:1–22. doi: 10.1101/cshperspect.a033092
43. Grüner S, Weber R, Peter D, Chung MY, Igreja C, Valkov E, et al. Structural motifs in eIF4G and 4E-BPs modulate their binding to eIF4E to regulate translation initiation in yeast. *Nucleic Acids Res.* (2018) 46:6893–908. doi: 10.1093/nar/gky542
44. Igreja C, Peter D, Weiler C, Izaurralde E. 4E-BPs require non-canonical 4E-binding motifs and a lateral surface of eIF4E to repress translation. *Nat Commun.* (2014) 5:1–14. doi: 10.1038/ncomms5790
45. Siomi MC, Siomi H, Sauer WH, Srinivasan S, Nussbaum RL, Dreyfuss G. FXR1, an autosomal homolog of the fragile X mental retardation gene. *EMBO J.* (1995) 14:2401–8. doi: 10.1002/j.1460-2075.1995.tb07237.x
46. Schenck A, Bardoni B, Moro A, Bagni C, Mandel JL. A highly conserved protein family interacting with the fragile X mental retardation protein (FMRP) and displaying selective interactions with FMRP-related proteins FXR1P and FXR2P. *Proc Natl Acad Sci USA.* (2001) 98:8844–9. doi: 10.1073/pnas.151231598
47. Erickson CA, Kaufmann WE, Budimirovic DB, Lachiewicz A, Haas-Givler B, Miller RM, et al. Best practices in fragile X syndrome treatment development. *Brain Sci.* (2018) 8:1–9. doi: 10.3390/brainsci8120224
48. Davenport MH, Schaefer TL, Friedmann KJ, Fitzpatrick SE, Erickson CA. Pharmacotherapy for fragile X syndrome: progress to date. *Drugs.* (2016) 76:431–45. doi: 10.1007/s40265-016-0542-y
49. Bagni C, Oostra BA. Fragile X syndrome: from protein function to therapy. *Am J Med Genet Part A.* (2013) 161A:2809–21. doi: 10.1002/ajmg.a.36241
50. Berry-Kravis EM, Lindemann L, Jønch AE, Apostol G, Bear MF, Carpenter RL, et al. Drug development for neurodevelopmental disorders: lessons learned from fragile X syndrome. *Nat Rev Drug Discov.* (2018) 17:280–99. doi: 10.1038/nrd.2017.221
51. Protic D, Salcedo-Arellano MJ, Dy JB, Potter LA, Hagerman RJ. New targeted treatments for fragile X syndrome. *Curr Pediatr Rev.* (2019) 15:251–8. doi: 10.2174/1573396315666190625110748
52. Castagnola S, Bardoni B, Maurin T. The search for an effective therapy to treat fragile X syndrome : dream or reality ? *Front Synaptic Neurosci.* (2017) 9:15. doi: 10.3389/fnsyn.2017.00015
53. Chiurazzi P, Pomponi MG, Willemsen R, Oostra BA, Neri G. *In vitro* reactivation of the FMR1 gene involved in fragile X syndrome. *Hum Mol Genet.* (1998) 7:109–13. doi: 10.1093/hmg/7.1.109
54. Chiurazzi P, Pomponi MG, Pietrobono R, Bakker CE, Neri G, Oostra BA. Synergistic effect of histone hyperacetylation and DNA demethylation

in the reactivation of the FMR1 gene. *Hum Mol Genet.* (1999) 8:2317–23. doi: 10.1093/hmg/8.12.2317
55. Tabolacci E, Palumbo F, Nobile V, Neri G. Transcriptional reactivation of the FMR1 gene. A possible approach to the treatment of the fragile X syndrome. *Genes.* (2016) 7:49. doi: 10.3390/genes7080049
56. Holoch D, Moazed D. RNA-mediated epigenetic regulation of gene expression. *Nat Rev Genet.* (2015) 16:71–84. doi: 10.1038/nrg3863
57. Rupaimoole R, Slack FJ. MicroRNA therapeutics: towards a new era for the management of cancer and other diseases. *Nat Rev Drug Discov.* (2017) 16:203–22. doi: 10.1038/nrd.2016.246
58. Suhl JA, Muddashetty RS, Anderson BR, Ifrim MF, Visootsak J, Bassell GJ, et al. A 3' untranslated region variant in FMR1 eliminates neuronal activity-dependent translation of FMRP by disrupting binding of the RNA-binding protein HuR. *Proc Natl Acad Sci USA.* (2015) 112:E6553–61. doi: 10.1073/pnas.1514260112
59. Wang C, Ge L, Wu J, Wang X, Yuan L. MiR-219 represses expression of dFMR1 in Drosophila melanogaster. *Life Sci.* (2019) 218:31–7. doi: 10.1016/j.lfs.2018.12.008
60. Pastori C, Peschansky VJ, Barbouth D, Mehta A, Silva JP, Wahlestedt C. Comprehensive analysis of the transcriptional landscape of the human FMR1 gene reveals two new long noncoding RNAs differentially expressed in Fragile X syndrome and Fragile X-associated tremor/ataxia syndrome. *Hum Genet.* (2014) 133:59–67. doi: 10.1007/s00439-013-1356-6
61. Shitik EM, Velmiskina AA, Dolskiy AA, Yudkin DV. Reactivation of FMR1 gene expression is a promising strategy for fragile X syndrome therapy. *Gene Ther.* (2020) 27:247–53. doi: 10.1038/s41434-020-0141-0
62. Gantois I, Bakker CE, Reyniers E, Willemsen R, D'Hooge R, De Deyn PP, et al. Restoring the phenotype of fragile X syndrome: insight from the mouse model. *Curr Mol Med.* (2001) 1:447–55. doi: 10.2174/1566524013363492
63. Zeier Z, Kumar A, Bodhinathan K, Feller JA, Foster TC, Bloom DC. Fragile X mental retardation protein replacement restores hippocampal synaptic function in a mouse model of fragile X syndrome. *Gene Ther.* (2009) 16:1122–9. doi: 10.1038/gt.2009.83
64. Gholizadeh S, Arsenault J, Xuan ICY, Pacey LK, Hampson DR. Reduced phenotypic severity following adeno-associated virus-mediated Fmr1 gene delivery in fragile X mice. *Neuropsychopharmacol.* (2014) 39:3100–11. doi: 10.1038/npp.2014.167
65. Xie N, Gong H, Suhl JA, Chopra P, Wang T, Warren ST. Reactivation of FMR1 by CRISPR/Cas9-mediated deletion of the expanded CGG-repeat of the fragile X chromosome. *PLoS ONE.* (2016) 11:e0165499. doi: 10.1371/journal.pone.0165499
66. Park CY, Halevy T, Lee DR, Sung JJ, Lee JS, Yanuka O, et al. Reversion of FMR1 methylation and silencing by editing the triplet repeats in fragile X iPSC-derived neurons. *Cell Rep.* (2015) 13:234–41. doi: 10.1016/j.celrep.2015.08.084
67. Lee B, Lee K, Panda S, Gonzales-Rojas R, Chong A, Bugay V, et al. Nanoparticle delivery of CRISPR into the brain rescues a mouse model of fragile X syndrome from exaggerated repetitive behaviours. *Nat Biomed Eng.* (2018) 2:497–507. doi: 10.1038/s41551-018-0252-8
68. Baker EK, Arpone M, Aliaga SM, Bretherton L, Kraan CM, Bui M, et al. Incomplete silencing of full mutation alleles in males with fragile X syndrome is associated with autistic features. *Mol Autism.* (2019) 10:21. doi: 10.1186/s13229-019-0271-7
69. Berry-Kravis E, Potanos K. Psychopharmacology in fragile X syndrome—present and future. *Ment Retard Dev Disabil Res Rev.* (2004) 10:42–8. doi: 10.1002/mrdd.20007
70. Schaefer TL, Davenport MH, Erickson CA. Emerging pharmacologic treatment options for fragile X syndrome. *Appl Clin Genet.* (2015) 8:75–93. doi: 10.2147/TACG.S35673
71. Berry-Kravis E, Des Portes V, Hagerman R, Jacquemont S, Charles P, Visootsak J, et al. Mavoglurant in fragile X syndrome: results of two randomized, double-blind, placebo-controlled trials. *Sci Transl Med.* (2016) 8:321ra5. doi: 10.1126/scitranslmed.aab4109
72. Youssef EA, Berry-Kravis E, Czech C, Hagerman RJ, Hessl D, Wong CY, et al. Effect of the mGluR5-NAM basimglurant on behavior in adolescents and adults with fragile X syndrome in a randomized, double-blind, placebo-controlled trial: fragXis phase 2 results. *Neuropsychopharmacol.* (2018) 43:503–12. doi: 10.1038/npp.2017.177
73. Hanson AC, Hagerman RJ. Serotonin dysregulation in fragile X syndrome: implications for treatment. *Intractable Rare Dis Res.* (2014) 3:110–7. doi: 10.5582/irdr.2014.01027
74. Lim C, Hoang ET, Viar KE, Stornetta RL, Scott MM, Zhu JJ. Pharmacological rescue of ras signaling, GluA1-dependent synaptic plasticity, and learning deficits in a fragile X model. *Genes Dev.* (2014) 28:273–89. doi: 10.1101/gad.232470.113
75. Greiss Hess L, Fitzpatrick SE, Nguyen DV, Chen Y, Gaul KN, Schneider A, et al. A randomized, double-blind, placebo-controlled trial of low-dose sertraline in young children with fragile X syndrome. *J Dev Behav Pediatr.* (2016) 37:619–28. doi: 10.1097/DBP.0000000000000334
76. Yoo KH, Burris JL, Gaul KN, Hagerman RJ, Rivera SM. Low-dose sertraline improves receptive language in children with fragile X syndrome when eye tracking methodology is used to measure treatment outcome. *Psychiatry Clin.* (2017) 1:1–8. doi: 10.15406/jpcpy.2017.07.00465
77. Maccarrone M, Rossi S, Bari M, De Chiara V, Rapino C, Musella A, et al. Abnormal mGlu 5 receptor/endocannabinoid coupling in mice lacking FMRP and BC1 RNA. *Neuropsychopharmacology.* (2010) 35:1500–9. doi: 10.1038/npp.2010.19
78. Jung KM, Sepers M, Henstridge CM, Lassalle O, Neuhofer D, Martin H, et al. Uncoupling of the endocannabinoid signalling complex in a mouse model of fragile X syndrome. *Nat Commun.* (2012) 3:1080. doi: 10.1038/ncomms2045
79. Heussler H, Cohen J, Silove N, Tich N, Bonn-Miller MO, Du W, et al. A phase 1/2, open-label assessment of the safety, tolerability, and efficacy of transdermal cannabidiol (ZYN002) for the treatment of pediatric fragile X syndrome. *J Neurodev Disord.* (2019) 11:16. doi: 10.1186/s11689-019-9277-x
80. Bergamaschi MM, Queiroz RHC, Chagas MHN, de Oliveira DCG, De Martinis BS, Kapczinski F, et al. Cannabidiol reduces the anxiety induced by simulated public speaking in treatment-naïve social phobia patients. *Neuropsychopharmacol.* (2011) 36:1219–26. doi: 10.1038/npp.2011.6
81. Busquets-Garcia A, Gomis-González M, Guegan T, Agustín-Pavón C, Pastor A, Mato S, et al. Targeting the endocannabinoid system in the treatment of fragile X syndrome. *Nat Med.* (2013) 19:603–7. doi: 10.1038/nm.3127
82. Osterweil EK, Chuang SC, Chubykin AA, Sidorov M, Bianchi R, Wong RKS, et al. Lovastatin corrects excess protein synthesis and prevents epileptogenesis in a mouse model of fragile X syndrome. *Neuron.* (2013) 77:243–50. doi: 10.1016/j.neuron.2012.01.034
83. Muscas M, Louros SR, Osterweil EK. Lovastatin, not simvastatin, corrects core phenotypes in the fragile X mouse model. *eNeuro.* (2019) 6:2019. doi: 10.1523/ENEURO.0097-19.2019
84. Cerezo-Guisado MI, García-Román N, García-Marín LJ, Alvarez-Barrientos A, Bragado MJ, Lorenzo MJ. Lovastatin inhibits the extracellular-signal-regulated kinase pathway in immortalized rat brain neuroblasts. *Biochem J.* (2007) 401:175–83. doi: 10.1042/BJ20060731
85. Siller SS, Broadie K. Neural circuit architecture defects in a Drosophila model of fragile X syndrome are alleviated by minocycline treatment and genetic removal of matrix metalloproteinase. *Dis Model Mech.* (2011) 4:673–85. doi: 10.1242/dmm.008045
86. Çaku A, Pellerin D, Bouvier P, Riou E, Corbin F. Effect of lovastatin on behavior in children and adults with fragile X syndrome: an open-label study. *Am J Med Genet Part A.* (2014) 164:2834–42. doi: 10.1002/ajmg.a.36750
87. Utari A, Chonchaiya W, Rivera SM, Schneider A, Hagerman RJ, Faradz SMH, et al. Side effects of minocycline treatment in patients with fragile X syndrome and exploration of outcome measures. *Am J Intellect Dev Disabil.* (2010) 115:433–43. doi: 10.1352/1944-7558-115.5.433
88. Leigh MJS, Nguyen DV, Mu Y, Winarni TI, Schneider A, Chechi T, et al. A randomized double-blind, placebo-controlled trial of minocycline in children and adolescents with fragile x syndrome. *J Dev Behav Pediatr.* (2013) 34:147–55. doi: 10.1097/DBP.0b013e318287cd17
89. Gantois I, Khoutorsky A, Popic J, Aguilar-Valles A, Freemantle E, Cao R, et al. Metformin ameliorates core deficits in a mouse model of fragile X syndrome. *Nat Med.* (2017) 23:674–7. doi: 10.1038/nm.4335
90. Gantois I, Popic J, Khoutorsky A, Sonenberg N. Metformin for treatment of fragile X syndrome and other neurological disorders. *Annu Rev Med.* (2019) 70:167–81. doi: 10.1146/annurev-med-081117-041238
91. Gkogkas CG, Khoutorsky A, Cao R, Jafarnejad SM, Prager-Khoutorsky M, Giannakas N, et al. Pharmacogenetic inhibition of eIF4E-dependent Mmp9

mRNA translation reverses fragile X syndrome-like phenotypes. *Cell Rep.* (2014) 9:1742–55. doi: 10.1016/j.celrep.2014.10.064
92. Hou L, Antion MD, Hu D, Spencer CM, Paylor R, Klann E. Dynamic translational and proteasomal regulation of fragile X mental retardation protein controls mGluR-dependent long-term depression. *Neuron.* (2006) 51:441–54. doi: 10.1016/j.neuron.2006.07.005
93. Dy ABC, Tassone F, Eldeeb M, Salcedo-Arellano MJ, Tartaglia N, Hagerman R. Metformin as targeted treatment in fragile X syndrome. *Clin Genet.* (2018) 93:216–22. doi: 10.1111/cge.13039
94. Monyak RE, Emerson D, Schoenfeld BP, Zheng X, Chambers DB, Rosenfelt C, et al. Insulin signaling misregulation underlies circadian and cognitive deficits in a Drosophila fragile X model. *Mol Psychiatry.* (2017) 22:1140–8. doi: 10.1038/mp.2016.51
95. Biag HMB, Potter LA, Wilkins V, Afzal S, Rosvall A, Salcedo-Arellano MJ, et al. Metformin treatment in young children with fragile X syndrome. *Mol Genet Genomic Med.* (2019) 7:e956. doi: 10.1002/mgg3.956
96. Protic D, Aydin EY, Tassone F, Tan MM, Hagerman RJ, Schneider A. Cognitive and behavioral improvement in adults with fragile X syndrome treated with metformin-two cases. *Mol Genet Genomic Med.* (2019) 7:e00745. doi: 10.1002/mgg3.745
97. Proteau-Lemieux M, Lacroix A, Galarneau L, Corbin F, Lepage JF, Çaku A. The safety and efficacy of metformin in fragile X syndrome: an open-label study. *Prog Neuro-Psychopharmacol Biol Psychiatry.* (2021) 110:110307. doi: 10.1016/j.pnpbp.2021.110307
98. Maurin T, Lebrigand K, Castagnola S, Paquet A, Jarjat M, Popa A, et al. HITS-CLIP in various brain areas reveals new targets and new modalities of RNA binding by fragile X mental retardation protein. *Nucleic Acids Res.* (2018) 46:6344–55. doi: 10.1093/nar/gky267
99. Androschuk A, He RX, Weber S, Rosenfelt C, Bolduc FV. Stress odorant sensory response dysfunction in drosophila fragile X syndrome mutants. *Front. Mol. Neurosci.* (2018) 11:242. doi: 10.3389/fnmol.2018.00242
100. Delhaye S, Bardoni B. Role of phosphodiesterases in the pathophysiology of neurodevelopmental disorders. *Mol Psychiatry.* (2021). doi: 10.1038/s41380-020-00997-9 [Epub ahead of print].
101. Choi CH, Schoenfeld BP, Weisz ED, Bell AJ, Chambers DB, Hinchey J, et al. PDE-4 inhibition rescues aberrant synaptic plasticity in Drosophila and mouse models of fragile X syndrome. *J Neurosci.* (2015) 35:396–408. doi: 10.1523/JNEUROSCI.1356-12.2015
102. Gurney ME, Nugent RA, Mo X, Sindac JA, Hagen TJ, Fox D 3rd, et al. Design and synthesis of selective phosphodiesterase 4D (PDE4D) allosteric inhibitors for the treatment of fragile X syndrome and other brain disorders. *J Med Chem.* (2019) 62:4884–901. doi: 10.1021/acs.jmedchem.9b00193
103. Maurin T, Melancia F, Jarjat M, Castro L, Costa L, Delhaye S, et al. Involvement of phosphodiesterase 2A activity in the pathophysiology of fragile X syndrome. *Cereb Cortex.* (2019) 29:3241–52. doi: 10.1093/cercor/bhy192
104. García-Font N, Martín R, Torres M, Oset-Gasque MJ, Sánchez-Prieto J. The loss of β adrenergic receptor mediated release potentiation in a mouse model of fragile X syndrome. *Neurobiol Dis.* (2019) 130:104482. doi: 10.1016/j.nbd.2019.104482
105. Gurney ME, Cogram P, Deacon RM, Rex C, Tranfaglia M. Multiple behavior phenotypes of the fragile-X syndrome mouse model respond to chronic inhibition of phosphodiesterase-4D (PDE4D). *Sci Rep.* (2017) 7:14653. doi: 10.1038/s41598-017-15028-x
106. Berry-Kravis EM, Harnett MD, Reines SA, Reese MA, Ethridge LE, Outterson AH, et al. Inhibition of phosphodiesterase-4D in adults with fragile X syndrome: a randomized, placebo-controlled, phase 2 clinical trial. *Nat Med.* (2021) 27:862–70. doi: 10.1038/s41591-021-01321-w
107. Paes D, Xie K, Wheeler DG, Zook D, Prickaerts J, Peters M. Inhibition of PDE2 and PDE4 synergistically improves memory consolidation processes. *Neuropharmacology.* (2021) 184:108414. doi: 10.1016/j.neuropharm.2020.108414
108. Bhat M, Robichaud N, Hulea L, Sonenberg N, Pelletier J, Topisirovic I. Targeting the translation machinery in cancer. *Nat Rev Drug Discov.* (2015) 14:261–78. doi: 10.1038/nrd4505
109. Laplante M, Sabatini DM. mTOR signaling in growth control and disease. *Cell.* (2012) 149:274–93. doi: 10.1016/j.cell.2012.03.017
110. Kaspar AA, Reichert JM. Future directions for peptide therapeutics development. *Drug Discov Today.* (2013) 18:807–17. doi: 10.1016/j.drudis.2013.05.011
111. Muttenthaler M, King GF, Adams DJ, Alewood PF. Trends in peptide drug discovery. *Nat Rev Drug Discov.* (2021) 20:309–25. doi: 10.1038/s41573-020-00135-8
112. D'Annessa I, Di Leva FS, La Teana A, Novellino E, Limongelli V, Di Marino D. Bioinformatics and biosimulations as toolbox for peptides and peptidomimetics design: where are we? *Front Mol Biosci.* (2020) 7:66. doi: 10.3389/fmolb.2020.00066
113. Moerke NJ, Aktas H, Chen H, Cantel S, Reibarkh MY, Fahmy A, et al. Small-molecule inhibition of the interaction between the translation initiation factors eIF4E and eIF4G. *Cell.* (2007) 128:257–67. doi: 10.1016/j.cell.2006.11.046
114. Wu M, Zhang C, Li XJ, Liu Q, Wanggou S. Anti-cancer effect of cap-translation inhibitor 4EGI-1 in human glioma U87 cells: involvement of mitochondrial dysfunction and ER stress. *Cell Physiol Biochem.* (2016) 40:1013–28. doi: 10.1159/000453158
115. Descamps G, Gomez-Bougie P, Tamburini J, Green A, Bouscary D, Maïga S, et al. The cap-translation inhibitor 4EGI-1 induces apoptosis in multiple myeloma through Noxa induction. *Br J Cancer.* (2012) 106:1660–7. doi: 10.1038/bjc.2012.139
116. Romagnoli A, Maracci C, D'Agostino M, Teana A La, Di Marino D. Targeting mTOR and eIF4E: a feasible scenario in ovarian cancer therapy. *Cancer Drug Resist.* (2021) 4:596–606. doi: 10.20517/cdr.2021.20
117. Santini E, Huynh TN, Longo F, Koo SY, Mojica E, D'Andrea L, et al. Reducing eIF4E-eIF4G interactions restores the balance between protein synthesis and actin dynamics in fragile X syndrome model mice. *Sci Signal.* (2018) 10:1–28. doi: 10.1126/scisignal.aan0665
118. Tan YS, Lane DP, Verma CS. Stapled peptide design: principles and roles of computation. *Drug Discov Today.* (2016) 21:1642–53. doi: 10.1016/j.drudis.2016.06.012
119. Lee ACL, Harris JL, Khanna KK, Hong JH. A comprehensive review on current advances in peptide drug development and design. *Int J Mol Sci.* (2019) 20:2383. doi: 10.3390/ijms20102383
120. Wells JA, McClendon CL. Reaching for high-hanging fruit in drug discovery at protein–protein interfaces. *Nature.* (2007) 450:1001–9. doi: 10.1038/nature06526
121. Fosgerau K, Hoffmann T. Peptide therapeutics: current status and future directions. *Drug Discov Today.* (2015) 20:122–8. doi: 10.1016/j.drudis.2014.10.003
122. Lenci E, Trabocchi A. Peptidomimetic toolbox for drug discovery. *Chem Soc Rev.* (2020) 49:3262–77. doi: 10.1039/D0CS00102C
123. Ryan P, Patel B, Makwana V, Jadhav HR, Kiefel M, Davey A, et al. Peptides, peptidomimetics, and carbohydrate–peptide conjugates as amyloidogenic aggregation inhibitors for Alzheimer's disease. *ACS Chem Neurosci.* (2018) 9:1530–51. doi: 10.1021/acschemneuro.8b00185
124. Ellert-Miklaszewska A, Poleszak K, Kaminska B. Short peptides interfering with signaling pathways as new therapeutic tools for cancer treatment. *Future Med Chem.* (2017) 9:199–221. doi: 10.4155/fmc-2016-0189
125. Marqus S, Pirogova E, Piva TJ. Evaluation of the use of therapeutic peptides for cancer treatment. *J Biomed Sci.* (2017) 24:21. doi: 10.1186/s12929-017-0328-x
126. Mabonga L, Kappo AP. Peptidomimetics: a synthetic tool for inhibiting protein–protein interactions in cancer. *Int J Pept Res Ther.* (2020) 26:225–41. doi: 10.1007/s10989-019-09831-5
127. Vercelli A, Biggi S, Sclip A, Repetto IE, Cimini S, Falleroni F, et al. Exploring the role of MKK7 in excitotoxicity and cerebral ischemia: a novel pharmacological strategy against brain injury. *Cell Death Dis.* (2015) 6:e1854. doi: 10.1038/cddis.2015.226
128. Baig MH, Ahmad K, Saeed M, Alharbi AM, Barreto GE, Ashraf GM, et al. Peptide based therapeutics and their use for the treatment of neurodegenerative and other diseases. *Biomed Pharmacother.* (2018) 103:574–81. doi: 10.1016/j.biopha.2018.04.025
129. Meredith ME, Salameh TS, Banks WA. Intranasal delivery of proteins and peptides in the treatment of neurodegenerative diseases. *AAPS J.* (2015) 17:780–7. doi: 10.1208/s12248-015-9719-7

130. De la Torre BG, Albericio F. Peptide therapeutics 2.0. *Molecules.* (2020) 25:2293. doi: 10.3390/molecules25102293
131. Chen RPY. From nose to brain: the promise of peptide therapy for Alzheimer's disease and other neurodegenerative diseases. *J Alzheimers Dis Parkinsonism*. (2017) 7:2–4. doi: 10.4172/2161-0460.1000314
132. Grüner S, Peter D, Weber R, Wohlbold L, Chung MY, Weichenrieder O, et al. The structures of eIF4E-eIF4G complexes reveal an extended interface to regulate translation initiation. *Mol Cell.* (2016) 64:467–79. doi: 10.1016/j.molcel.2016.09.020
133. Peter D, Weber R, Köne C, Chung MY, Ebertsch L, Truffault V, et al. Mextli proteins use both canonical bipartite and novel tripartite binding modes to form eIF4E complexes that display differential sensitivity to 4E-BP regulation. *Genes Dev.* (2015) 29:1835–49. doi: 10.1101/gad.269068.115
134. Saxton RA, Sabatini DM. mTOR signaling in growth, metabolism, and disease. *Cell.* (2017) 168:960–76. doi: 10.1016/j.cell.2017.02.004
135. Cingolani LA, Goda Y. Actin in action: the interplay between the actin cytoskeleton and synaptic efficacy. *Nat Rev Neurosci.* (2008) 9:344–56. doi: 10.1038/nrn2373
136. Irwin SA, Galvez R, Greenough WT. Dendritic spine structural anomalies in fragile-X mental retardation syndrome. *Cereb Cortex.* (2000) 10:1038–44. doi: 10.1093/cercor/10.10.1038
137. Chen LY, Rex CS, Babayan AH, Kramár EA, Lynch G, Gall CM, et al. Physiological activation of synaptic Rac>PAK (p-21 Activated Kinase) signaling is defective in a mouse model of fragile X syndrome. *J Neurosci.* (2010) 30:10977. doi: 10.1523/JNEUROSCI.1077-10.2010
138. Castets M, Schaeffer C, Bechara E, Schenck A, Khandjian EW, Luche S, et al. FMRP interferes with the Rac1 pathway and controls actin cytoskeleton dynamics in murine fibroblasts. *Hum Mol Genet.* (2005) 14:835–44. doi: 10.1093/hmg/ddi077
139. Chen B, Chou HT, Brautigam CA, Xing W, Yang S, Henry L, et al. Rac1 GTPase activates the WAVE regulatory complex through two distinct binding sites. *Elife.* (2017) 6:e29795. doi: 10.7554/eLife.29795
140. Chen Z, Borek D, Padrick SB, Gomez TS, Metlagel Z, Ismail AM, et al. Structure and control of the actin regulatory WAVE complex. *Nature.* (2010) 468:533–8. doi: 10.1038/nature09623
141. Rottner K. WAVE regulatory complex. *Curr Biol.* (2021) 31:R496–552. doi: 10.1016/j.cub.2021.01.086
142. Song YK, Guo H, Barengo N, Naora H. Inhibition of ovarian cancer growth by a tumor-targeting peptide that binds eukaryotic translation initiation factor 4E. *Clin Cancer Res.* (2009) 15:4336–47. doi: 10.1158/1078-0432.CCR-08-2924
143. Masse M, Glippa V, Saad H, Le Bloas R, Gauffeny I, Berthou C, et al. An eIF4E-interacting peptide induces cell death in cancer cell lines. *Cell Death Dis.* (2014) 5:e1500–8. doi: 10.1038/cddis.2014.457
144. Gallagher EE, Menon A, Chmiel AF, Deprey K, Kritzer JA, Garner AL. A cell-penetrant lactam-stapled peptide for targeting eIF4E protein-protein interactions. *Eur J Med Chem.* (2020) 205:112655. doi: 10.1016/j.ejmech.2020.112655
145. Lama D, Liberatore AM, Frosi Y, Nakhle J, Tsomaia N, Bashir T, et al. Structural insights reveal a recognition feature for tailoring hydrocarbon stapled-peptides against the eukaryotic translation initiation factor 4E protein. *Chem Sci.* (2019) 10:2489–500. doi: 10.1039/C8SC03759K
146. Lama D, Quah ST, Verma CS, Lakshminarayanan R, Beuerman RW, Lane DP, et al. Rational optimization of conformational effects induced by hydrocarbon staples. *Sci Rep.* (2013) 3:1–10. doi: 10.1038/srep03451
147. Lama D, Verma CS. Deciphering the mechanistic effects of eIF4E phosphorylation on mRNA-cap recognition. *Protein Sci.* (2020) 29:1373–86. doi: 10.1002/pro.3798
148. Gentilucci L, Marco R De, Cerisoli L. Chemical modifications designed to improve peptide stability : incorporation of non-natural amino acids, pseudo-peptide bonds, and cyclization. *Curr Pharm Des.* (2010) 16:3185–203. doi: 10.2174/138161210793292555
149. Greco I, Molchanova N, Holmedal E, Jenssen H, Hummel BD, Watts JL, et al. Correlation between hemolytic activity, cytotoxicity and systemic in vivo toxicity of synthetic antimicrobial peptides. *Sci Rep.* (2020) 10:13206. doi: 10.1038/s41598-020-69995-9
150. Gupta S, Kapoor P, Chaudhary K, Gautam A, Kumar R, Raghava GPS. Peptide toxicity prediction. *Methods Mol Biol.* (2015) 1268:143–57. doi: 10.1007/978-1-4939-2285-7_7

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A Longitudinal Study of Parent-Child Interactions and Language Outcomes in Fragile X Syndrome and Other Neurodevelopmental Disorders

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Difficulties with pragmatic language (i.e., language in social contexts, such as conversational ability) are a noted characteristic of the language profiles of both fragile X syndrome (FXS) and autism spectrum disorder (ASD), conditions which show significant phenotypic overlap. Understanding the origins and developmental course of pragmatic language problems in FXS and other developmental conditions associated with language impairment is a critical step for the development of targeted interventions to promote communicative competence across the lifespan. This study examined pragmatic language in the context of parent-child interactions in school-age children with FXS (who did and did not meet ASD criteria on the ADOS; $n = 85$), idiopathic ASD ($n = 32$), Down syndrome (DS; $n = 38$), and typical development (TD; $n = 39$), and their parents. Parent-child communicative interactions were examined across multiple contexts, across groups, and in relationship to pragmatic language outcomes assessed 2 years later. Results showed both overlapping and divergent patterns across the FXS-ASD and idiopathic ASD child and parent groups, and also highlighted key differences in pragmatic profiles based on situational context, with more pragmatic language difficulties occurring for both ASD groups in less structured interactions. Differences in parental language styles during parent-child interactions were associated with child language outcomes, likely reflecting the complex interplay of discourse style inherent to a parent, with the inevitable influence of child characteristics on parent language as well. Together, findings help delineate the dynamic and multifactorial nature of impaired pragmatic skills among children with FXS and other neurodevelopmental disorders associated with language impairment, with potential implications for the development of targeted interventions for pragmatic communication skills.

Keywords: pragmatic language, social communication, fragile X syndrome, autism spectrum disorder, parent-child interaction, broad autism phenotype, longitudinal outcomes

INTRODUCTION

Pragmatic language refers to the use of language in social contexts and draws on a broad range of linguistic, paralinguistic, neuropsychological, and social skills (1–10). For instance, successful conversations (a key pragmatic skill) require an individual to take turns; introduce, maintain, and change topics; demonstrate an awareness and understanding of conversational partners; and keep up with conversational demands and expectations (8). This dynamic and complex set of language skills also serves a pivotal role in supporting social interactions, and when impaired, can seriously undercut social functioning (6, 11).

Difficulties in pragmatic communication are a hallmark of autism spectrum disorder (ASD), a neurodevelopmental disability characterized by the presence of social and communicative impairments and patterns of restricted and repetitive behaviors or interests (12). Similar deficits are also observed in fragile X syndrome (FXS), a single-gene disorder caused by a mutation in the *FMR1* gene and the most common single-gene disorder associated with ASD (13–16). Importantly, areas of pragmatic language overlap (and divergence) have been noted between individuals with FXS-associated ASD (FXS-ASD) (based on meeting ASD criteria on the Autism Diagnostic Observation Schedule; ADOS) and idiopathic ASD (ASD-O) (17). For example, research has shown that both groups use non-contingent (i.e., off-topic) and perseverative (i.e., repetitive) language at higher rates than children with other types of neurodevelopmental disabilities, such as Down syndrome (DS) and FXS without significant ASD symptomatology (FXS-Only; FXS-O) (17–21). However, prior research has also found higher rates of initiations and lower rates of non-responsiveness in boys with FXS-ASD compared to boys with ASD-O (17). Evidence of similarities in pragmatic language phenotypes in FXS-ASD and ASD-O is potentially significant for understanding the shared etiology of such impairments, whereas knowledge of both similarities and differences can also inform pragmatic language interventions with these groups where targeted therapies can be implemented. Thus, clarifying the specific pragmatic needs of individuals with FXS with and without ASD symptomatology, and in relation to ASD-O, has important implications for targeting and advocating for more effective treatments in FXS.

Importantly, little is known about the development of pragmatic language in FXS compared to idiopathic ASD, and how conversational context and communication partner may contribute to patterns of pragmatic strengths and weaknesses—all critical questions to address in order to understand the extent of similarities in pragmatic profiles in FXS-ASD and ASD-O, and whether they may stem from common origins. Moreover, studies comparing pragmatic impairments in other genetically-based neurodevelopmental disorders (e.g., DS and FXS-O) relative to FXS-ASD and ASD-O are limited, leaving unclear the specificity of pragmatic impairments in these different populations (17, 22, 23). For example, pragmatic language impairments in DS may manifest differently due to a prominent discrepancy between social motivation and language difficulties (24–26). Evidence suggests that although children with DS commit fewer pragmatic violations compared to children with ASD and FXS (27–29), this group tends to show difficulty with topic elaboration, introduction, and maintenance (29, 30). They also tend to use increased stereotyped language compared to mental age matched controls (31, 32). Direct comparison of pragmatic language in DS and FXS with and without ASD symptomatology, and idiopathic ASD is needed to understand the specificity or potential overlap of such impairments across conditions, and to clarify the role of global developmental delay and intellectual disability (ID) vs. ASD-specific symptomatology in the pragmatic difficulties observed across groups. Indeed, while pragmatic language difficulties have been documented in ID, the nature of these impairments differs from what is seen in ASD, as social communication difficulties in ID are not thought to exceed the individual's broader profile of abilities and functional capacities (33).

This study adopted a cross-population, longitudinal design to comprehensively characterize pragmatic skills in school-age children with FXS who did and did not meet ASD criteria on the ADOS (FXS-ASD, FXS-O), idiopathic ASD (ASD-O), DS, and younger controls with typical development (TD), and to examine pragmatics across structured and unstructured conversational interactions with their parents. Importantly, parent-child communicative interactions served as the focus of analysis because parents are often the child's primary conversational partner, serving as a key source of language input throughout childhood. This is particularly true among children with developmental disabilities, as children with significant cognitive and language delays are less likely to extend the range of their communication partners throughout development relative to their typically developing counterparts (34, 35). Indeed, as children enter middle childhood and adolescence, they begin to master conversational skills, including appropriate referencing, increased turn-taking, adapting speaking style to conversational partner and context, and cohesion (36). Beyond this, the social demands of conversational contexts become more complex and nuanced over this developmental period (37), with an increased reliance on cognitive systems that are often significantly impacted in children with both ASD and ID. Studies of TD have also provided clear evidence of the critical role that parent discourse style can play in child language and social-emotional development [e.g., (38–41)]. In atypical development, parental discourse during parent-child interactions similarly has the potential to influence a child's language outcomes (42–45). Not surprisingly, overall parent-child synchrony and parental responsiveness during interactions is associated with better language outcomes in children with ASD (43, 46). Maternal responsiveness is also associated with child language in FXS (47). In addition, better pragmatic language in mothers of children with ASD appears related to better expressive language skills in 2–4-year-old children (45). Together, findings highlight the important ways in which parental language styles can influence children's language development, but also certainly reflect a bidirectional relationship in which parent and child language features influence each other in complex ways that have yet to be delineated in populations where pragmatics are centrally impacted. Most prior research addressing such questions has focused on early infancy and toddlerhood, leaving important

questions unanswered regarding the school-age children, when continued and increasingly complex opportunities for language learning (particularly pragmatic language skills) and skill mastery occur (28, 48).

Examining parent-child interactions is also of particular significance in these groups given evidence of subtle pragmatic language differences among parents of individuals with ASD, which are believed to reflect genetic liability to ASD (20, 21, 49–53). Together with certain personality styles (e.g., social reticence, rigid personality), pragmatic differences comprise a constellation of traits that mirror the defining characteristics of ASD and are referred to collectively as the Broad Autism Phenotype (BAP) (20, 53). Features of the BAP (and pragmatic differences in particular) have also been observed among mothers of individuals with FXS, who are carriers of the *FMR1* gene in its premutation state (20, 54). Some evidence suggests that parent pragmatic language differences are associated with pragmatic language development in children with ASD and FXS (45, 55, 56). To date, however, no study has directly examined the potential association between parent and child pragmatic language during parent-child interactions in school-age children, and the majority of studies that have looked at parent-child relationships have relied on global measures of language from separate conversational contexts in parents and their children, rather than the parent-child interactions in which such associations might be most effectively studied. Data examining the interrelationships between parents and children during conversational interactions has the potential to identify key pragmatic features in both communicative partners that can serve as important pathways for targeting parent-mediated interventions in order to most effectively address the child's particular needs. Similarly, delineating the complexity of these relationships may also highlight BAP features that serve as protective factors on child language development (e.g., eliciting more language by indulging tangents; adhering to routine-based interactions). Thus, this approach can also clarify parents' pragmatic strengths in order to maximize these in treatment and optimize child outcomes.

This study applied a detailed hand-coding (i.e., manual, turn-by-turn coding) system adapted from Roberts et al. (29) and Martin et al. (17), and previously used to describe pragmatic language in children with FXS, ASD, DS, and TD during semi-structured interactions with a trained examiner, to characterize pragmatic language across groups during two distinct parent-child interaction contexts, with a subgroup studied over time. In addition to group comparisons, analyses examined interrelationships between parent and child pragmatic phenotypes, and how such features related to child pragmatic outcomes 2 years later. The overarching goals of this study were to delineate the complex pragmatic language phenotypes associated with different neurodevelopmental conditions and identify the influence of parent-child interaction styles on child language outcomes across these groups. Specific aims were as follows:

Aim 1: To compare child pragmatic profiles across groups during parent-child interactions. Key pragmatic language features were compared across groups. Sex differences were also examined. Based on the extant literature, alongside underlying difficulties with social cognition observed in ASD, it was predicted that the ASD groups (FXS-ASD and ASD-O) would demonstrate greater pragmatic deficits relative to the comparison groups, with most profound differences noted in key areas of non-contingent and perseverative language (20–22, 28, 29, 57). It was further predicted that individuals in the ASD groups would demonstrate better pragmatic abilities during structured interaction as compared to unstructured interaction given the greater social demands inherent in unstructured situations, and evidence suggesting that unstructured discourse contexts are most challenging for individuals with ASD [e.g., (58)].

Aim 2: To compare parent pragmatic profiles across groups during parent-child interactions. Given evidence of pragmatic language differences in the broad autism phenotype and among a subgroup of carriers of the *FMR1* premutation, and alongside weaknesses in social cognition, it was predicted that parents of children in the ASD groups (FXS-ASD and ASD-O) would exhibit greater differences in pragmatic behaviors, including non-contingent language, which has been reported in prior literature. The effects of context were predicted to mirror the same trends that were expected for children.

Aim 3: To examine interrelationships between parent and child language. It was expected that non-contingent and perseverative language would be interrelated in parents and children in all groups, but specifically the ASD groups.

Aim 4: To identify key features of parent-child interactions that predict child pragmatic outcomes 2 years later, across diagnostic groups. Overall parent and child responsiveness during parent-child interactions was predicted to influence child pragmatic language outcomes across groups (44, 47, 59).

MATERIALS AND METHODS

Parent and child participants were recruited as part of a larger study on pragmatic language development. Both males and females were included in all groups except the idiopathic ASD group, which included only males due to the aims of the larger longitudinal study from which these data were drawn (see **Table 1** for participant characteristics). Although the majority of the parent sample consisted of mothers, fathers participated in 17 cases across groups (3 fathers participated with males with FXS-ASD, 5 with males with ASD-O, 3 with males with FXS-O, 2 with males with DS, 2 with males with TD, and 1 with a female with TD). Mothers in the FXS groups were all confirmed carriers of the *FMR1* premutation. The total parent sample therefore consisted of 17 father-child dyads and 177 mother-child dyads. Sixteen sets of siblings were included in which a parent participated more than one time with a different child in the same diagnostic group. To address this, the effect of family was examined in statistical analyses and is reported in the analysis plan below. All siblings and parents were included in the overall sample.

Inclusion criteria for the broader longitudinal study [described in greater detail in Ref. (17)] included English as a primary language, using three or more words in an

TABLE 1 | Participant characteristics.

Males

Variable	FXS-ASD, M (SD) *n* = 39	FXS-O, M (SD) *n* = 10	ASD-O, M (SD) *n* = 32	DS, M (SD) *n* = 21	TD, M (SD) *n* = 19
Chronological age	10.9 (2.3)[a]	9.6 (3.2)[a,b]	8.6 (2.8)[b]	**10.9 (2.1)[a]**	4.6 (1.1)[c]
Non-verbal mental age[a]	5.1 (0.41)[a]	**5.3 (0.63)[a]**	7.0 (3.5)[b]	5.1 (0.41)[a]	5.2 (1.2)[a]
Receptive vocabulary age[b]	5.9 (1.4)[a,b]	**5.9 (1.6)[a,b]**	6.3 (2.7)[a]	5.9 (1.4)[b]	6.0 (1.6)[a]
Expressive vocabulary age[c]	**5.2 (1.0)[a,c]**	**5.2 (1.0)[a,b]**	6.0 (2.3)[b]	5.4 (1.3)[c]	5.6 (1.6)[d]
MLU[d]	**3.4 (0.76)[a]**	**3.9 (0.75)[a]**	4.1 (1.1)[b]	3.1 (0.77)[a]	4.8 (0.73)[b]
Autism severity[e]	6.6 (1.5)[a]	2.5 (1.0)[b]	7.6 (1.9)[c]	1.4 (0.55)[b]	1.6 (0.69)[b]
Parent education level	16.0 (2.4)[a]	15.9 (1.3)[a]	16.0 (2.3)[a]	16.6 (2.3)[a]	16.0 (2.5)[a]

Females

Variable	FXS-ASD, M (SD) *n* = 11	FXS-O, M (SD) *n* = 25	ASD-O, M (SD) *n* = 32	DS, M (SD) *n* = 17	TD, M (SD) *n* = 20
Chronological age	9.1 (3.8)[a]	9.0 (3.7)[a]		**9.1 (2.2)[a]**	5.4 (2.4)[b]
Non-verbal mental age[a]	5.4 (0.95)[a]	**7.0 (2.7)[b]**		5.1 (0.71)[a]	6.2 (2.6)[a,b]
Receptive vocabulary age[b]	7.4 (3.4)[a]	**8.2 (3.5)[a]**		4.7 (1.6)[b,c]	6.5 (3.1)[a,c]
Expressive vocabulary age[c]	**6.4 (2.0)[a,b]**	**8.4 (3.8)[b]**		4.7 (1.5)[a]	6.2 (2.4)[a]
MLU[d]	**4.3 (1.2)[a]**	**4.8 (1.1)[a]**		3.3 (1.0)[b]	5.0 (1.4)[a]
Autism severity[e]	6.1 (1.7)[a]	2.1 (0.86)[b]		1.8 (0.62)[b,c]	1.5 (0.67)[c]
Parent education level	15.0 (1.6)[a]	16.0 (2.1)[a]		15.4 (2.3)[a]	16.0 (2.8)[a]

FXS-ASD, fragile X syndrome with autism spectrum disorder based on the ADOS; ASD-O, ASD only; FXS-O, FXS only; DS, Down syndrome; TD, typical development; M, Mean; SD, Standard Deviation. Different superscripts within a row indicate significant differences at $p < 0.05$. If groups share the same letter, differences were not significant. Bolded items indicate significant sex differences.

[a] *Leiter International Performance Scale-Revised.*

[b] *Peabody Picture Vocabulary Test-III or IV.*

[c] *Expressive Vocabulary Test.*

[d] *Mean length of utterance in morphemes.*

[e] *Autism Diagnostic Observation Schedule.*

utterance, having no history of developmental or language delays in the TD group, and having the *FMR1* full mutation in the FXS group. Participants who failed a hearing screening with a threshold >30 dB HL in the better ear across 500, 1,000, 2,000, and 4,000 Hz were excluded from the study. Participants in the TD and DS groups were screened for ASD using the Autism Diagnostic Observation Schedule (ADOS) (60). Any subject in the TD or DS groups who met criteria for autism or autism spectrum on the ADOS were excluded from the broader longitudinal study, while subjects with idiopathic ASD or FXS who met ADOS criteria for autism or autism spectrum were included in one of the ASD groups. Of note, the Autism Diagnostic Interview, Revised (ADI-R) (61) was administered whenever possible, though due to time constraints was available on only 56% of the sample. Therefore, the ADOS was used for group classification. Participants in the ASD-O group had all previously received a clinical diagnosis of ASD. Because subjects received multiple administrations of the ADOS as part of the larger longitudinal research study, average ADOS severity scores were calculated to determine ASD classification [(62); see further description in Ref. (17)]. All examiners who administered the ADOS satisfied reliability criteria set forth by the test authors. Eight participants were excluded from the present study (and are thus not included in **Table 1**) because the dyad either interacted for <5 min and/or did not speak long enough to generate the minimum number of total turns required for analyses (20 turns for each 5-min task). For the structured task, this included 1 male with FXS-ASD, 2 females with FXS-ASD, and 1 female with DS. For the unstructured task, this included 2 males with ASD-O, 1 female with FXS-ASD, and 1 female with FXS-O.

Participants enrolled in this study were administered a battery of language, cognitive, and clinical-behavioral measures in addition to the ADOS. The larger study from which these data were drawn implemented a rolling enrollment schedule, where participants were eligible to enroll at any point during the 5-year study period. Participants who enrolled later in the study did not complete later timepoints of longitudinal data collection, but were nonetheless included at time 1 to increase power for group comparison data. The total sample size for longitudinal analyses included: 28 boys and 8 girls with FXS-ASD, 5 boys and 21 girls FXS-O, 11 boys with ASD-O, 14 boys and 10 girls with DS, and 9 boys and 9 girls with TD.

The study battery was administered in a quiet room, either in the child's home, school, or at a research laboratory. Testing sessions were audiotaped using a digital audio recorder (Marantz PMD670) and videotaped using a SONY Digital 8 camcorder (Model DCR-TVR27). All procedures were approved by University of North Carolina at Chapel Hill and Northwestern University Institutional Review Boards.

Cognitive and Structural Language Abilities

The Leiter International Performance Scale-Revised (63) was used to assess non-verbal cognitive abilities. Structural language measures included expressive vocabulary, receptive vocabulary, and expressive syntactic complexity. These skills were assessed using the Expressive Vocabulary Test [EVT; (64)], Peabody Picture Vocabulary Test-3rd or 4th edition [PPVT; (65, 66)], and mean length of utterance (MLU) in morphemes (67), respectively. MLU was based on ADOS language samples, which occurred at the same time point as the parent-child interactions and were computed using Systematic Analysis of Language Transcripts [SALT; (68)] software.

Parent-Child Interactions

Parent-child interactions included one structured and one relatively unstructured interactive task, each lasting 5-min.

Structured Interaction

Parents and children were asked to plan a "fun day out" together. For this task, the examiner provided five different picture cards and instructed parent-child dyads to discuss where they would like to go, who they would like to go with, what they would need to bring, how they would get there, and what they would like to do/see at the destination. The picture cards involved scenes from the zoo, park/playground, pool, beach, and shopping center.

Unstructured Interaction

Parents and children engaged in a "free play" task in which examiners presented a box of toys (e.g., flashlight, kaleidoscope, prism, rainbow glasses, periscope, picture cards) and provided the parent and child with only minimal instructions to look at the toys together, so that interactions could unfold in a relatively unstructured manner.

Pragmatic Language

Pragmatic language was measured both at study entry, and 2 years following the initial parent-child interaction, using the following measures.

Comprehensive Assessment of Spoken Language (CASL)

The Pragmatic Judgment subtest of the CASL (69) is a standardized test measure that requires children to state how they would respond in various social situations (e.g., "How would you greet an unfamiliar adult?"). Consistent with past research [e.g., (70)], age equivalence scores were used in analysis.

Pragmatic Rating Scale-School Age (PRS-SA)

The Pragmatic Rating Scale-School Age [PRS-SA; (71)] is a pragmatic language rating system designed to characterize a range of pragmatic language abilities based on semi-naturalistic, conversational interactions administered as part of the ADOS. The PRS-SA includes 34 operationally defined verbal and non-verbal pragmatic language features rated 0, 1, or 2 (indicating presence and degree of impairment for each item, with scores of 2 indicating greatest impairment) by independent coders from videotaped recordings. Coders were never provided with participant diagnostic status, but were also unlikely to be fully blinded given facial dysmorphology that occurs in DS and often FXS. Reliability for the PRS-SA based on the larger study from which these data were drawn, as well as ongoing studies in our lab which include samples not included in the current study, is 78.4%. A subset of these files were consensus coded, and the consensus coded scores were used in analyses.

Transcription and Coding

Parent-child language samples were transcribed verbatim by transcribers who achieved morpheme-to-morpheme agreement rates of 80% or higher. Coders (coding system described below) were similarly trained to a minimum of 80% training reliability across three separate files. As was the case with the PRS-SA, fully blinded status of coders was not possible given the use of video. Nine percent of all coded files were also randomly checked for reliability. Intraclass correlation coefficients [ICCs; (72)] were as follows for children: non-contingent language (0.94), perseveration (0.87), initiations (0.98), and non-responsiveness (0.87). ICCs were as follows for parents: non-contingent language (0.84), perseveration (0.68), initiations (0.99), and non-responsiveness (1.0). ICCs from 0.5 to 0.75 are considered to represent moderate agreement, 0.75–0.9 to represent good agreement, and >0.9 to represent excellent agreement (73). The reliability files were subsequently consensus coded. Coding was based on parent-child dyadic turns. A parent-child dyadic turn was defined as either one back-and-forth parent-child exchange (e.g., parent speaks and child responds or vice versa) and/or a comment/question that was met with a non-response by the other conversational partner for a period of at least 3 s. Each parent-child task was coded separately, by coders who were blind to group status.

Pragmatic Language Coding System

Pragmatic language skills during the structured and unstructured interaction tasks were coded using a system adapted from Roberts et al. (29) and Martin et al. (17), which examines discrete aspects of pragmatic language, such as contingency of conversational partners' contributions, initiations and responsiveness, and perseverative language. Pragmatic codes are further described in **Table 2**. Unintelligible utterances were excluded from calculations.

Analysis Plan

Analyses of group differences in children and parents controlled for child non-verbal mental age, expressive and receptive vocabulary, and mean length of utterance (MLU), given significant differences across groups and because of the impact of general cognition and structural language on pragmatic abilities [e.g., (74)]. There were no significant differences in parental

TABLE 2 | Pragmatic coding system.

Variable	Definition	Example (s)*	Calculation
Non-contingent Language	Off-topic or tangential turns	Par: What is this? **Ch: Good to see you.**	Total non-contingent turns/total codable dyadic turns
Perseveration	Excessive repetition of words, phrases, sentences, or topics	Par: What's in there? Ch: I ain't telling. Par: I wanna see. Ch: She always lets me look. Nobody else. Par: I'm gonna cry. I'm gonna cry. Ch: She's supposed to let me look. Par: I'm gonna cry. Do you want me to cry? Ch: She always let's me look in here. Par: Okay, but do you want me to cry? Ch: No. Par: Well I am going to cry if you don't let me look. … Ch: She told me I can look in here. She is a student and she told me I can look. Par: I am going to cry now. C: That's what she said. P: I think I'm going to cry now.	Total perseverative turns/total codable dyadic turns
Initiations	Self-initiated turns	Par: I think we would need to take the car to the zoo. Ch: Yea, it would be a long walk if we didn't drive. **What animals will be there?**	Total initiations/total codable dyadic turns
Non-response	Failure to respond when response is required (within 3 s)	Par: What do we do with this toy? **Ch: No response.**	Total non-responses/total codable dyadic turns

**With the exception of perseveration, which demonstrates examples for both parent and child, examples demonstrate each variable in the child, with the same coding guidelines used for parents.*

education across groups where data were available ($n = 55$; ps > 0.80) and thus this was not included as a covariate. Given that participants were classified based only the ADOS, partial correlations for participants with FXS (regardless of ASD classification status) were conducted using the four child outcome variables and ASD severity. Results based on these analyses are presented as a complementary table (see **Table 3.4**) and allow for examination of relationships with ASD severity as a continuous measure. Across analyses (with the exception of linear regressions for interrelationships and correlational analyses described in pragmatic longitudinal outcomes), planned comparisons were conducted even when overall models were not significant, given the novelty of this data and to guard against Type 2 errors (75). In addition, given multiple tests and a rather small dataset, Bonferroni corrected analyses were conducted for child and parent MANCOVAs following initial analyses with no adjustments, to address the possibility of false discovery. Only findings that withstood Bonferroni corrections are reported in the text below. These findings are also denoted in each of the corresponding tables. However, because adjustment assumes that a Type 1 error is of more serious concern than a Type 2 error (76), and given the uniqueness of these data and difficulty ascertaining rare populations such as FXS (particularly for longitudinal studies), the danger of missing effects was of greater concern. Therefore, findings without adjustments for the MANCOVAs are also reported in tables. To further aid in interpretation of data, Cohen's d effect sizes are also provided for all analyses examining group differences (see **Tables 3.3**, **4.3**). With the exception of pragmatic longitudinal outcomes which include both significant and marginal findings given small sample size, only significant results are described. The corresponding tables for each analysis present the remaining statistical results.

Pragmatic Language in Children

Pragmatic language during parent-child interactions was analyzed using a series of multivariate analyses of covariance (MANCOVAs). Group differences were examined, as well as within group sex differences (except for the ASD-O group, where data on females were not available). The effect of context (structured vs. unstructured parent-child interaction task) was evaluated using repeated measures ANCOVA to investigate a diagnosis by context interaction.

Pragmatic Language in Parents

To characterize parent pragmatic profiles across groups during parent-child interactions, analyses followed the same plan that was used for the analysis of child data described above (i.e., group comparisons were based on child diagnosis and sex). To address concerns related to non-independence (i.e., a small subset of cases included the same parent with a different child, $n = 16$), a linear mixed model was conducted for each outcome variable with participant nested within family. None of these models were significant and the random effect for diagnosis in these cases was essentially zero. This suggested that the nesting of individuals within families did not result in non-independence. While it is possible that there was an insufficient number of family cases included in the overall sample to fully test this effect, generalized linear models (GLMs) were additionally conducted with and

without siblings and the findings were compared to each other. Because there were few differences between these two models, and there was no effect of family status in the mixed model, the GLMs reported below include all available participants regardless of family status in order to increase sample size and power.

Parent-Child Interrelationships

To examine patterns of parent-child relationships, and limit the number of correlations examined, a principal component analysis (PCA) with a one component solution was conducted for parent and child groups separately with all language variables (i.e., non-contingent language, non-responsiveness, initiation, perseveration) included. The PCA resulted in a component for the child group explaining 40.09% of the variance, with standardized loadings of 0.26 for initiation, 0.37 for non-responsiveness, 0.84 for non-contingent language, and 0.84 for perseveration. The parent component explained 35.37% of the variance, with standardized loadings of −0.39 for initiation, 0.32 for non-responsiveness, 0.82 for non-contingent language, and 0.70 for perseveration. The component score for each subject was then used in exploratory Pearson correlations within each group, with separate analyses for sex and context, resulting in a total of 18 correlation models.

Pragmatic Language Outcomes

To identify the relationship between parent-child interactions (including both child and parent language during interactions) at baseline and child pragmatic outcomes 2-years later across diagnostic groups, a series of partial correlations were conducted. The variables included each of the parent and child language variables explored in the group differences and interrelationship analyses above (e.g., parent and child non-contingent language), as well as PRS-SA and CASL scores at time 3 (two years later). Covariates included the baseline measure of the outcome variable being explored, which was mean centered (e.g., PRS-SA scores at baseline served as the covariate for relationships with longitudinal PRS-SA scores). Of note, all parents of boys with ASD-O and girls with FXS-ASD included in longitudinal analyses received scores of zero for non-responsiveness, resulting in insufficient variability within these groups to examine these relationships in a meaningful way and relationships with parental non-responsiveness for these two groups were therefore not examined.

RESULTS

Pragmatic Language in Children

Boys

As indicated in **Table 3.1**, the model for non-contingent language was significant in both tasks, driven by boys with FXS-ASD and ASD-O compared to all other groups. There were no significant effects of context. Similar findings emerged for perseveration, such that boys with FXS-ASD and ASD-O used higher rates of perseveration compared to other groups during structured interactions. Non-responsiveness was significant during both interaction types. This effect was driven by higher rates of non-responsiveness among boys with ASD-O compared to all other groups. Boys with FXS-ASD, ASD-O, DS, and TD were more non-responsive during the unstructured interactions compared to the structured interactions.

Girls

Girls with FXS-ASD used more non-contingent language relative to all other groups (see **Table 3.2**). There were no significant effects for context. Similar findings emerged for perseveration and were primarily driven by girls with FXS-ASD and FXS-O. There was no significant effect for context overall. Girls with FXS-ASD were less responsive during structured and unstructured interactions than girls in all other groups. There was no significant effect for context.

Sex Differences

There were significant sex differences in rates of non-responsiveness between boys and girls with FXS-ASD [$F_{(1,46)} = 13.8$, $p < 0.01$; $F_{(1,46)} = 3.8$, $p < 0.01$] across both interaction contexts, with girls showing higher rates of non-responsiveness than boys.

Pragmatic Language in Parents

Parents of Boys

Parents interacting with their children with FXS-ASD and ASD-O used more non-contingent language across both contexts compared to parents from other groups (see **Table 4.1**). Parents of children with ASD-O and FXS-ASD were also more perseverative than parents interacting with children with DS. Rates of initiations and non-responsiveness were not significant for either context.

Parents of Girls

The FXS-ASD parent group used higher rates of non-contingent language relative to the other groups (see **Table 4.2**). Higher rates of non-contingent language were observed during unstructured interaction relative to the structured interaction in parents of individuals with FXS-O and DS. Non-responsiveness did not occur often enough during structured interactions for a valid model estimate to be derived.

Parent-Child Interrelationships

Correlation coefficients from the PCA-derived components are summarized in **Table 5** and reported in detail here. In boys and girls with FXS-ASD, the components were associated between the parent and child groups in the unstructured interactions (rs > 0.61, $ps < 0.01$), and there were no significant associations in the structured interactions ($ps > 0.08$). No significant associations emerged in the FXS-O groups ($ps > 0.28$) or DS groups ($ps > 0.16$). For boys with ASD-O, a significant relationship emerged between the parent and child groups during structured interactions ($r = 0.46$, $p = 0.01$) but not in unstructured interactions ($r = 0.05$, $p = 0.79$). In girls with TD, a significant association emerged in unstructured interactions ($r = 48$, $p = 0.03$). There were no associations in boys with TD ($ps > 0.20$).

TABLE 3.1 | Group differences in pragmatic language in males.

Structured interaction						
Pragmatic language	**Group F**	**FXS-ASD, M(SE)**	**FXS-O, M(SE)**	**ASD-O, M(SE)**	**DS, M(SE)**	**TD, M(SE)**
Non-contingent	$F_{(4,112)} = 10.7^{***\dagger}$	0.18 (0.02)^{a}	0.08 (0.03)^{b}	0.16 (0.02)^{a}	0.04 (0.02)^{b}	0.07 (0.02)^{b}
Perseveration	$F_{(4,112)} = 6.1^{***\dagger}$	0.07 (0.009)^{a}	0.03 (0.02)^{b}	0.05 (0.01)^{a,b}	0.005 (0.01)^{c}	0.02 (0.01)^{c}
Initiations	$F_{(4,112)} = 1.3$	0.34 (0.02)^{a,b}	0.42 (0.04)^{a}	0.31 (0.03)^{b}	0.33 (0.03)^{a,b}	0.34 (0.03)^{a,b}
Non-responsiveness	$F_{(4,112)} = 7.3^{***\dagger}$	***0.01 (0.006)^{a}***	0.01 (0.01)^{a}	**0.05 (0.007)^{b}**	**0.003 (0.008)^{a}**	**0.02 (0.009)^{a}**
Unstructured interaction						
Pragmatic language	**Group F**	**FXS-ASD, M(SE)**	**FXS-O, M(SE)**	**ASD-O, M(SE)**	**DS, M(SE)**	**TD, M(SE)**
Non-contingent	$F_{(4,112)} = 16.2^{***\dagger}$	*0.24 (0.02)^{a}*	0.07 (0.03)^{b}	0.18 (0.02)^{a}	0.05 (0.03)^{b}	0.06 (0.03)^{b}
Perseveration	$F_{(4,112)} = 4.2^{**}$	0.11 (0.01)^{a}	0.04 (0.03)^{b}	0.07 (0.02)^{a,b}	0.03 (0.02)^{b}	0.03 (0.02)^{b}
Initiations	$F_{(4,112)} = 0.19$	0.49 (0.02)^{a}	0.53 (0.04)^{a}	0.49 (0.03)^{a}	0.48 (0.03)^{a}	0.49 (0.04)^{a}
Non-responsiveness	$F_{(4,112)} = 6.7^{***\dagger}$	***0.04 (0.02)^{a}***	0.03 (0.03)^{a}	**0.14 (0.02)^{b}**	**0.04 (0.02)^{a}**	**0.08 (0.02)^{a}**

Context effect

Pragmatic language	**Group F**
Non-contingent	$F_{(4,112)} = 1.4$
Perseveration	$F_{(4,112)} = 0.38$
Initiations	$F_{(4,112)} = 0.45$
Non-responsiveness	$F_{(4,112)} = 2.6^{*}$

FXS-ASD, fragile X syndrome with autism spectrum disorder based on the ADOS; ASD-O, ASD only; FXS-O, FXS only; DS, Down syndrome; TD, typical development. Adjusted Means (M) and Standard Errors (SE). Different superscripts within a row indicate significant differences at $p < 0.05$. If groups share the same letter, differences were not significant. Bolded items indicate significant differences between contexts within a diagnostic group. Italicized items indicated significant within group sex differences.
$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, †indicates significance following Bonferroni corrections.

Pragmatic Language Outcomes

FXS-ASD

Increased rates of child non-contingent language ($r = 0.51$, $p = 0.007$) and perseveration ($r = 0.42$, $p = 0.03$) in the structured interaction were associated with greater pragmatic difficulties in boys with FXS-ASD 2 years later, as measured by the PRS-SA (see **Figure 1A**). Increased rates of parent non-responsiveness ($r = 0.34$, $p = 0.08$) and parent perseveration ($r = -0.35$, $p = 0.07$) in the structured interaction were marginally associated with greater pragmatic difficulties in boys based on the PRS-SA. In contrast, among girls, increased rates of child non-contingent language in the unstructured interaction were marginally associated with better pragmatic outcomes based on the PRS-SA ($r = -0.68$, $p = 0.09$; see **Figure 1B**). No other significant or marginal relationships emerged (ps > 0.10) (e.g., see **Figure 1C**).

FXS-O

Increased rates of child perseveration ($r = -0.96$, $p = 0.04$) and child non-responsiveness ($r = 0.98$, $p = 0.02$) during structured parent-child interactions were associated with greater pragmatic difficulties 2 years later, as measured by the CASL, in boys with FXS-O. There were no significant relationships when examining the parent-child interactions and child longitudinal outcomes based on the PRS-SA in this group (ps > 0.17). In girls with FXS-O, higher rates of parent non-contingent language in the structured interaction ($r = -0.48$, $p = 0.03$) were associated with greater pragmatic difficulties, as measured by the CASL, 2 years later. Marginal relationships emerged between parent initiations in the unstructured interaction and pragmatic outcomes based on the PRS-SA ($r = 0.41$, $p = 0.07$). No other significant or marginal relationships emerged for girls with FXS-O on either the CASL or PRS-SA (ps > 0.14) (e.g., see **Figure 1C**).

ASD-O

Partial correlations in boys with ASD-O revealed that child non-contingent language in the unstructured interaction ($r = -0.90$, $p = 0.001$) was associated with lower (i.e., worse) child CASL scores 2 years later (see **Figure 1D**). Parent initiations in the unstructured interaction were negatively related to child pragmatic outcomes based on the CASL ($r = -0.68$, $p = 0.05$). Parent non-responsiveness in unstructured interaction was also related to better pragmatic outcomes on the CASL ($r = 0.78$, $p = 0.01$). No other significant relationships emerged (ps > 0.24) (e.g., see **Figure 1C**).

DS

In boys with DS, a marginal relationship emerged between child non-responsiveness in the unstructured interaction and pragmatic outcomes based on the PRS-SA ($r = 0.49$, $p = 0.09$).

TABLE 3.2 | Group differences in pragmatic language in females.

Structured interaction					
Pragmatic language	**Group F**	**FXS-ASD, M(SE)**	**FXS-O, M(SE)**	**DS, M(SE)**	**TD, M(SE)**
Non-ontingent	$F_{(3,65)} = 6.2^{**\dagger}$	0.17 (0.02)^{a}	0.06 (0.02)^{b}	0.04 (0.02)^{b}	0.05 (0.02)^{b}
Perseveration	$F_{(3,65)} = 5.4^{**\dagger}$	0.05 (0.01)^{a}	0.03 (0.007)^{a,c}	−0.003 (0.009)^{b}	0.007 (0.008)^{b,c}
Initiations	$F_{(3,65)} = 0.85$	0.38 (0.05)^{a}	0.41 (0.03)^{a}	0.37 (0.04)^{a}	0.41 (0.04)^{a}
Non-responsiveness	$F_{(3,65)} = 5.4^{***\dagger}$	*0.06 (0.009)^{a}*	0.01 (0.006)^{b}	0.002 (007)^{b}	0.02 (0.007)^{b}
Unstructured interaction					
Pragmatic language	**Group F**	**FXS-ASD, M(SE)**	**FXS-O, M(SE)**	**DS, M(SE)**	**TD, M(SE)**
Non-contingent	$F_{(3,65)} = 8.4^{***\dagger}$	0.15 (0.02)^{a}	0.06 (0.02)^{b}	0.03 (0.02)^{b}	0.04 (0.02)^{b}
Perseveration	$F_{(3,65)} = 6.1^{**\dagger}$	0.10 (0.02)^{a}	0.07 (0.01)^{a,b}	−0.002 (0.02)^{c}	0.04 (0.02)^{b,c}
Initiations	$F_{(3,65)} = 1.9$	0.61 (0.05)^{a}	0.61 (0.03)^{a}	0.52 (0.04)^{b}	0.52 (0.04)^{a,b}
Non-responsiveness	$F_{(3,65)} = 3.3^{*\dagger}$	*0.13 (0.02)^{a}*	0.05 (0.02)^{b}	0.03 (0.02)^{b}	0.05 (0.02)^{b}

Context effect

Pragmatic language	**Group F**
Non-contingent	$F_{(3,65)} = 0.49$
Perseveration	$F_{(3,65)} = 1.5$
Initiations	$F_{(3,65)} = 1.6$
Non-responsiveness	$F_{(3,65)} = 0.58$

FXS-ASD, fragile X syndrome with autism spectrum disorder based on the ADOS; FXS-O, FXS only; DS, Down syndrome; TD, typical development. Adjusted Means (M) and Standard Errors (SE).Different superscripts within a row indicate significant differences at p < 0.05. If groups share the same letter, differences were not significant. Italicized items indicated significant within group sex differences.

$^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, †indicates significance following Bonferroni corrections.

Child perseveration in the structured interaction ($r = -0.53$, $p = 0.08$) and parent non-responsiveness in the unstructured interaction ($r = -0.54$, $p = 0.07$) were also marginally related to CASL scores 2 years later, such that higher rates of child perseveration and parent non-responsiveness were associated with greater pragmatic difficulties 2 years later. In girls with DS, increased parent initiations in the structured interaction ($r = -0.71$, $p = 0.02$) and perseveration in the unstructured interaction ($r = -0.63$, $p = 0.05$) were related to poorer pragmatic outcomes, as measured by the CASL. However, increased child non-contingent language (rs > 0.74, *p*s < 0.03) during both contexts and increased child perseveration in the unstructured context ($r = 0.78$, $p = 0.01$) was associated with higher (i.e., worse) PRS-SA scores (see **Figures 1A,B**). Marginal relationships also emerged between child initiations in the structured interaction and pragmatic outcomes on the PRS-SA ($r = -0.61$, $p = 0.08$) (i.e., increased initiations during parent-child interactions associated with better PRS-SA scores 2 years later). No other significant or marginal relationships emerged (*p*s > 0.11) (e.g., see **Figure 1C**).

TD

Partial correlations revealed a significant relationship between parent perseveration in the unstructured context ($r = 0.89$, $p = 0.003$) and CASL scores 2 years later in boys with TD, such that higher rates of parent perseveration were associated with better pragmatic outcomes. Higher rates of parent non-contingent language in the structured context ($r = -0.72$, $p = 0.05$) and non-responsiveness in the unstructured context ($r = -0.71$, $p = 0.05$) were also associated with better pragmatic scores on the PRS-SA in boys with TD. Marginal relationships emerged between child perseveration in the unstructured context in boys and pragmatic outcomes based on the CASL ($r = 0.66$, $p = 0.08$). Higher rates of child non-responsiveness in the unstructured context were associated with better pragmatic scores on the CASL in girls with TD ($r = 0.83$, $p = 0.02$), and increased parent perseveration in the unstructured contexts was marginally associated with more pragmatic difficulties on the PRS-SA ($r = 0.67$, $p = 0.07$). No other significant or marginal relationships emerged (*p*s > 0.10).

DISCUSSION

This study applied a detailed pragmatic coding system to characterize parent and child pragmatic skills during conversational interactions in children with fragile X syndrome who did and did not meet ASD criteria on the ADOS (FXS-ASD, FXS-O), idiopathic autism spectrum disorder (ASD-O), Down

TABLE 3.3 | Effect sizes (Cohen's d) for pragmatic language in males and females.

Structured interaction/unstructured interaction						**Structured interaction/unstructured interaction**			
Non-contingent language	**Males**					**Females**			
	FXS-ASD	**FXS-O**	**ASD-O**	**DS**	**TD**	**FXS-ASD**	**FXS-O**	**DS**	**TD**
FXS-ASD	–	0.9/1.4	0.17/0.5	1.2/1.5	1.0/1.4	–	1.2/1.7	1.8/1.5	1.5/1.4
FXS-O	–	–	0.7/1.0	0.4/0.16	0.1/0.08	–	–	0.2/0.5	0.1/0.3
ASD-O	–	–	–	1.2/1.1	0.88/1	–	–	–	–
DS	–	–	–	–	0.3/0.07	–	–	–	0.1/0.1
Perseveration	**Males**					**Females**			
	FXS-ASD	**FXS-O**	**ASD-O**	**DS**	**TD**	**FXS-ASD**	**FXS-O**	**DS**	**TD**
FXS-ASD	–	0.7/1.0	0.4/0.5	1.2/1.1	0.97/1.1	–	0.6/0.6	1.5/1.4	1.3/0.8
FXS-O	–	–	0.4/0.3	0.5/0.1	0.2/0.1	–	–	0.9/1.1	0.7/0.4
ASD-O	–	–	–	0.9/0.4	0.6/0.4	–	–	–	–
DS	–	–	–	–	0.3/0.0	–	–	–	0.3/0.5
Initiations	**Males**					**Females**			
	FXS-ASD	**FXS-O**	**ASD-O**	**DS**	**TD**	**FXS-ASD**	**FXS-O**	**DS**	**TD**
FXS-ASD	–	0.7/0.3	0.2/0.0	0.08/0.8	0.0/0.0	–	0.2/0.0	0.06/0.6	0.2/0.5
FXS-O	–	–	0.7/0.3	0.7/0.4	0.6/0.3	–	–	0.3/0.6	0.0/0.6
ASD-O	–	–	–	0.1/0.06	0.2/0	–	–	–	–
DS	–	–	–	–	0.08/0.07	–	–	–	0.2/0.0
Non-responsiveness	**Males**					**Females**			
	FXS-ASD	**FXS-O**	**ASD-O**	**DS**	**TD**	**FXS-ASD**	**FXS-O**	**DS**	**TD**
FXS-ASD	–	0.0/0.09	1.1/0.85	0.2/0	0.3/0.4	–	1.7/0.9	2.1/1.4	1.3/1.1
FXS-O	–	–	1.1/1.0	0.2/0.1	0.3/0.6	–	–	0.3/0.3	0.3/0.1
ASD-O	–	–	–	1.2/0.9	0.8/0.5	–	–	–	–
DS	–	–	–	–	0.5/0.5	–	–	–	0.6/0.2

Effect sizes were calculated based on adjusted mean, standard error, and individual group sample size. Cells with – indicate redundant effect sizes, within-group effect sizes that are not calculated, or effect sizes that could not be calculated (e.g., no ASD-O group females).

TABLE 3.4 | Partial correlations based on ASD-severity and pragmatic language in FXS.

	Non-contingent		**Perseveration**		**Nonresponse**		**Initiations**	
	Structured	**Unstructured**	**Structured**	**Unstructured**	**Structured**	**Unstructured**	**Structure**	**Unstructured**
Males	**0.44****	**0.46****	**0.30***	**0.39****	0.002	0.01	−0.02	0.02
Females	**0.53****	**0.36***	0.16	0.05	**0.5****	***0.36****	−0.17	−0.00

*Results based on correlational analyses generally follow the same trends as those observed based on group classification status; Bolded items indicate statistical significance; Italicized items indicated significant within group sex differences. *p < 0.05, **p < 0.01.*

syndrome (DS), and typical development (TD), as well as interrelationships and pragmatic language outcomes 2 years later. Sex differences were also examined within each diagnostic group, with the exception of the ASD-O group because girls were not included in the broader longitudinal study from which these data were drawn. Results suggest both important areas of overlap and divergence in pragmatic skills and patterns of association, across child and parent groups, and in different conversational contexts. The FXS-ASD and ASD-O groups showed particular similarities, with some important exceptions. Parent-child analyses also suggest associations indicative of reciprocal interactions in the ways that parents and children use pragmatic skills during both structured and unstructured conversations, although patterns differed across groups, and direction of influence in these associations remains unclear (though likely to be highly bidirectional). In what follows, results

TABLE 4.1 | Group differences in pragmatic language in parents of males.

Structured interaction

Pragmatic language	Group F	FXS-ASD, M(SE)	FXS-O, M(SE)	ASD-O, M(SE)	DS, M(SE)	TD, M(SE)
Non-contingent	$F_{(4, 112)} = 3.2^{*\dagger}$	**0.02 (0.004)^{a}**	0.02 (0.008)^{a,b,c}	**0.02 (0.005)^{a,c}**	0.002 (0.006)^{b}	**0.006 (0.006)^{b,c}**
Perseveration	$F_{(4, 112)} = 0.34$	**0.01 (0.005)^{a}**	**0.02 (0.009)^{a}**	0.006 (0.005)^{a}	0.007 (0.007)^{a}	0.009 (0.007)^{a}
Initiations	$F_{(4, 112)} = 0.11$	0.95 (0.008)^{a}	0.94 (0.02)^{a}	0.95 (0.009)^{a}	0.95 (0.01)^{a}	0.95 (0.01)^{a}
Non-responsiveness	$F_{(4, 112)} = 0.34$	0.006 (0.007)^{a}	−0.002 (0.01)^{a}	0.002 (0.008)^{a}	0.01 (0.009)^{a}	0.003 (0.01)^{a}

Unstructured interaction

Pragmatic language	Group F	FXS-ASD, M(SE)	FXS-O, M(SE)	ASD-O, M(SE)	DS, M(SE)	TD, M(SE)
Non-contingent	$F_{(4, 112)} = 4.9^{**\dagger}$	**0.06 (0.007)^{a}**	0.01 (0.01)^{b}	**0.04 (0.008)^{a,b}**	0.02 (0.01)^{b}	**0.01 (0.01)^{b}**
Perseveration	$F_{(4, 112)} = 2.9^{*\dagger}$	**0.05 (0.01)^{a}**	**0.03 (0.02)^{a,b}**	0.05 (0.01)^{a}	0.004 (0.01)^{b}	0.01 (0.02)^{a,b}
Initiations	$F_{(4, 112)} = 0.97$	0.92 (0.01)^{a}	0.92 (0.02)^{a}	0.92 (0.01)^{a}	0.91 (0.02)^{a}	0.88 (0.02)^{a}
Non-responsiveness	$F_{(4, 112)} = 1.4$	0.01 (0.01)^{a}	0.003 (0.02)^{a}	0.01 (0.01)^{a}	0.04 (02)^{a}	0.04 (0.02)^{a}

Context effect

Pragmatic language	Group F
Non-contingent	$F_{(4, 112)} = 2.2^{\wedge}$
Perseveration	$F_{(4, 112)} = 3.0^{*}$
Initiations	$F_{(4, 112)} = 1.4$
Non-responsiveness	$F_{(4, 112)} = 0.79$

Parent groups based on child status; FXS-ASD, fragile X syndrome with autism spectrum disorder based on the ADOS; ASD-O, ASD only; FXS-O, FXS only; DS, Down syndrome; TD, typical development. Adjusted Means (M) and Standard Errors (SE). Different superscripts within a row indicate significant differences at $p < 0.05$. If groups share the same letter, differences were not significant. Bolded items indicate significant differences between contexts within a diagnostic group.
$^{\wedge}p < 0.10$, $^{*}p < 0.05$, $^{**}p < 0.01$, †indicates significance following Bonferroni correction.

are discussed in greater detail across each of the primary sets of analyses.

Child Group and Sex Differences

In line with prior research where the child interacted with a trained examiner (17, 29), and consistent with predictions, boys and girls with FXS-ASD, and boys with ASD-O, demonstrated higher rates of non-contingent language during structured (i.e., "fun day out" task) and unstructured (i.e., "free play" task) parent-child interactions relative to all other groups. The findings for perseverative language generally paralleled the same trends that emerged for non-contingent responses (although the model for perseveration during unstructured interactions did not withstand multiple-comparison correction and should be interpreted with caution). In other words, boys and girls with FXS-ASD were more perseverative during both structured and unstructured interactions relative to all other groups. Boys with ASD-O were significantly more perseverative during structured interaction than boys with TD and DS. Although this finding was not significant during unstructured interaction, the same pattern of differences emerged. Together, these findings add to a growing body of research that supports non-contingent and perseverative language as central components of the pragmatic phenotype associated with ASD with and without FXS (17–19, 29, 77).

In contrast to these areas of overlap, we observed differences between the two groups of boys with ASD, particularly with respect to lower rates of initiations and responsiveness in boys with ASD-O compared to FXS-ASD (although the model for initiations did not withstand multiple-comparison correction and should be interpretated with caution). Nonetheless, this finding builds on those reported from a prior study looking at examiner-child interactions in an overlapping sample of participants (17), suggesting a more pervasive pattern that extends across different types of conversational interactions and partners. These findings may reflect important differences in underlying social motivations, as the ability to initiate conversation represents a core pragmatic impairment among boys with ASD-O (17, 78–81). Similarly, the ability to be responsive also represents a clear pragmatic deficit for boys with ASD-O and girls with FXS-ASD. This finding was expected and adds to the growing body of evidence pointing to non-responsiveness as a major factor in the pragmatic phenotype of idiopathic ASD (17, 82, 83). It also reveals an important sex difference between boys and girls with FXS-ASD, as boys did not show difficulty with responsiveness. Notably, social anxiety and hyperarousal are major factors in the social phenotype of girls with FXS-ASD (84), and could be contributing to these findings.

Important differences were also observed in boys and girls with FXS-O and DS. Consistent with recent research,

TABLE 4.2 | Group differences in pragmatic language in parents of females.

Structured interaction					
Pragmatic language	**Group F**	**FXS-ASD, M(SE)**	**FXS-O, M(SE)**	**DS, M(SE)**	**TD, M(SE)**
Non-contingent	$F_{(3,65)} = 2.6$^	0.02 (0.006)[a]	0.004 (0.004)[b]	0.004 (0.005)[b]	0.01 (0.005)[a,b]
Perseveration	$F_{(3,65)} = 0.76$	0.005 (0.003)[a]	0.001 (0.002)[a]	0.00 (0.002)[a]	0.003 (0.002)[a]
Initiations	$F_{(3,65)} = 0.77$	0.94 (0.02)[a]	0.95 (0.01)[a]	0.96 (0.01)[a]	0.94 (0.01)[a]
Non-responsiveness	–	0.00 (0.00)	0.00 (0.00)	0.00 (00)	0.00 (0.00)
Unstructured interaction					
Pragmatic language	**Group F**	**FXS-ASD, M(SE)**	**FXS-O, M(SE)**	**DS, M(SE)**	**TD, M(SE)**
Non-contingent	$F_{(3,65)} = 1.2$	0.04 (0.01)[a]	0.02 (0.007)[a]	0.02 (0.01)[a]	0.02 (0.008)[a]
Perseveration	$F_{(3,65)} = 1.8$	0.03 (0.01)[a]	0.008 (0.006)[b]	0.01 (0.008)[a,b]	0.009 (0.007)[a,b]
Initiations	$F_{(3,65)} = 0.29$	0.93 (0.02)[a]	0.90 (0.02)[a]	0.91 (0.02)[a]	0.91 (0.02)[a]
Non-responsiveness	$F_{(3,65)} = 0.13$	0.02 (0.02)[a]	0.02 (0.01)[a]	0.009 (01)[a]	0.01 (0.01)[a]
Context effect					
Pragmatic language	**Group F**				
Non-contingent	$F_{(3,65)} = 0.58$				
Perseveration	$F_{(3,65)} = 1.4$				
Initiations	$F_{(3,65)} = 0.77$				
Non-responsiveness	$F_{(3,65)} = 0.13$				

Parent groups based on child status; FXS-ASD, fragile X syndrome with autism spectrum disorder based on the ADOS; FXS-O, FXS only; DS, Down syndrome; TD, typical development. Adjusted Means (M) and Standard Errors (SE). Different superscripts within a row indicate significant differences at $p < 0.05$. If groups share the same letter, differences were not significant. ^$p < 0.10$.

neither of these groups had difficulty with non-contingent language relative to typically developing controls (17, 19, 29, 70), which suggests that increased non-contingent language use may be more unique to individuals in the ASD groups and is not alone attributable to general cognitive delay. In fact, contingent discourse represented a relative strength among boys and girls with FXS-O and DS. Additionally, inasmuch as previous investigations have suggested that non-contingent responding is typical of children with FXS [e.g., (85, 86)], it is important to note that defining the presence vs. absence of ASD symptomatology appears to make an important difference.

Whereas, boys and girls with DS did not show any difficulty with perseveration, girls with FXS-O were more perseverative than girls with DS or TD, and there were no differences in rates of perseveration between boys with FXS-O and ASD-O, who both showed more perseveration than DS and TD groups. These findings are slightly different from what was observed in a similar sample during examiner-child interactions, where Martin et al. (17) found no evidence of increased perseveration in the FXS-O groups. This important context difference highlights perseveration as a key behavior to consider in FXS, independent of ASD symptomatology.

Parent Group and Sex Differences

The pragmatic profiles of parents generally followed the same trends that occurred for children. Parents interacting with girls and boys with FXS-ASD, and boys with ASD-O, showed higher rates of non-contingent language in both contexts relative to the other groups. Additionally, these same parent groups used higher rates of perseverative language during unstructured parent-child interactions (although the model for perseveration in parents of girls did not withstand multiple-comparison correction and should be interpretated with caution). It is possible that these findings were child-driven, as these parent groups have children who show the very same types of pragmatic weaknesses. However, these types of pragmatic differences have also been described as important features of the BAP (20, 21, 50, 53). A subgroup of parents of children with ASD and carriers of the *FMR1* premutation have been shown to use more tangential (including topic preoccupation) or off-topic language, during conversational interactions with examiners (20).

These findings suggest that non-contingent and perseverative language represent an important component of the pragmatic phenotype associated with ASD with and without FXS, in both affected and unaffected individuals. This potential pragmatic *signature* appears to cut across diagnostic boundaries in children and parents, and may help define the etiologies of pragmatic

TABLE 4.3 | Effect sizes (Cohen's d) for pragmatic language in parents.

Structured interaction/unstructured interaction						Structured interaction/unstructured interaction			
Non-contingent language	**Parents of males**					**Parents of females**			
	FXS-ASD	**FXS-O**	**ASD-O**	**DS**	**TD**	**FXS-ASD**	**FXS-O**	**DS**	**TD**
FXS-ASD	–	0/1.2	0/0.5	0.7/0.9	0.6/1.2	–	0.8/0.6	0.8/0.5	0.5/0.6
FXS-O	–	–	0/0.7	0.7/0.2	0.6/0	–	–	0/0	0.3/0
ASD-O	–	–	–	0.7/0.4	0.5/0.7	–	–	–	–
DS	–	–	–	–	0.2/0.2	–	–	–	0.3/0
Perseveration	**Parents of males**					**Parents of females**			
	FXS-ASD	**FXS-O**	**ASD-O**	**DS**	**TD**	**FXS-ASD**	**FXS-O**	**DS**	**TD**
FXS-ASD	–	0.5/0.3	0.1/0.4	0.1/0.7	0.1/0.5	–	0.4/0.7	0.5/0.6	0.1/0.7
FXS-O	–	–	0.6/0.3	0.6/0.5	0.5/0.3	–	–	0.1/0.1	0.3/0
ASD-O	–	–	–	0.03/0.7	0.1/0.6	–	–	–	–
DS	–	–	–	–	0.04/0.2	–	–	–	0.3/0
Initiations	**Parents of males**					**Parents of females**			
	FXS-ASD	**FXS-O**	**ASD-O**	**DS**	**TD**	**FXS-ASD**	**FXS-O**	**DS**	**TD**
FXS-ASD	–	0.2/0	0/0	0/0.1	0/0.6	–	0.2/0.3	0.3/0.6	0/0.3
FXS-O	–	–	0/0	0/0.1	0/0.5	–	–	0.2/0.1	0.2/0.1
ASD-O	–	–	–	0/0.1	0/0.6	–	–	–	–
DS	–	–	–	–	0/0.3	–	–	–	0.5/0
Non-responsiveness	**Parents of males**					**Parents of females**			
	FXS-ASD	**FXS-O**	**ASD-O**	**DS**	**TD**	**FXS-ASD**	**FXS-O**	**DS**	**TD**
FXS-ASD	–	0.2/0.1	0.1/0	0.1/0.4	0.1/0.4	–	–/0	–/0.2	–/0.2
FXS-O	–	–	0.1/0.1	0.3/0.5	0.1/0.5	–	–	–/0.2	–/0.2
ASD-O	–	–	–	0.2/0.4	0/0.4	–	–	–	–
DS	–	–	–	–	0.2/0	–	–	–	–/0

Effect sizes were calculated based on adjusted mean, standard error, and individual group sample size. Cells with – indicate redundant effect sizes, within-group effect sizes that are not calculated, or effect sizes that could not be calculated (e.g., no ASD-O group in parents of females).

impairment in FXS and ASD. These findings also underscore the need to consider parental language style in the development of targeted parent-child interventions, as parents may be genetically predisposed to certain language styles that are exacerbated in the context of interactions with their children.

Interrelationships Between Parent and Child Pragmatic Behaviors

A number of interesting patterns of parent-child interrelationships emerged across groups for the PCA-derived components. Non-contingent language and perseveration contributed most significantly to both the parent and child components (>0.70), with initiation and non-responsiveness contributing less so with standardized loadings <0.40. Higher ratings on both components indicated more difficulty with pragmatic language, specifically more non-contingent language and perseveration, during the dyadic interactions.

Examining these components, significant positive parent-child correlations emerged for boys and girls with FXS-ASD, boys with ASD-O, and girls with TD. For boys and girls with FXS-ASD and boys with ASD-O, increased rates of non-contingent language and perseveration were observed in analyses of group differences, with similar patterns emerging in their parents, likely reflecting similar pragmatic weaknesses in these dyads. The positive association further indicates that, within groups, parents with more severe weaknesses were more likely to have children with more severe weaknesses. This finding is potentially indicative of genetic influences, although environmental effects cannot be ruled out. The significant association for girls with TD and their parents is perhaps less notable given that pragmatic weaknesses were not found for these groups, although the correlation still indicates that such behaviors, even if relatively infrequent, are related in these dyads.

The bidirectional nature of these associations is important to consider, as parent and child language patterns are certainly

interdependent. For example, a parent could be off-topic because their child was off-topic to begin with or vice versa, or non-contingent language could occur as part of a parents' natural attempt to redirect their child to a particular topic. It may also be that because of a child's tendency to respond in an off-topic or socially inappropriate way during conversational interactions, parents may be more prone to change the topic or redirect the conversation back to what was originally being discussed. In sum, parent and child behaviors are highly interrelated and in important ways driven by the other's behaviors. The bidirectional nature of these relationships should be further studied in designs capable of teasing out causal direction, such as parent training intervention studies, which may help to identify the most effective response patterns for supporting the development of more contingent discourse in these dyadic interactions.

Together, these findings emphasize the ways in which parent and child contributions to an unfolding communicative interaction are intricately interrelated. It is clear that even if parents and children have genetic predispositions toward certain language styles, pragmatic features do not occur in isolation; instead, they are dynamic and bidirectional. Children who have difficulty with these types of pragmatic skills in particular could benefit from targeted interventions aimed at improving specific parent-child interaction patterns.

Finally, the lack of significant parent-child interrelationships in boys or girls with FXS-O and DS, and boys with TD, may be due to fewer atypical pragmatic behaviors being present in these groups overall. This may also suggest that dyads in these groups employed different pragmatic styles, perhaps inconsistently, that did not significantly relate to one another.

TABLE 5 | Interrelationships between parent and child pragmatic language.

		Child component	
		Structured context	**Unstructured context**
Parent Component	FXS-ASD boys ($n = 39$)	0.27	**0.62*****
	FXS-ASD girls ($n = 11$)	0.47	**0.75****
	FXS-O boys ($n = 10$)	0.22	0.30
	FXS-O girls (n =25)	0.07	−0.07
	ASD-O boys ($n = 32$)	**0.46****	0.05
	DS boys ($n = 21$)	0.15	0.09
	DS girls ($n = 17$)	0.18	0.17
	TD boys ($n = 19$)	−0.01	0.27
	TD girls ($n = 20$)	0.18	**0.48***

*Bolded items indicate statistical significance; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.*

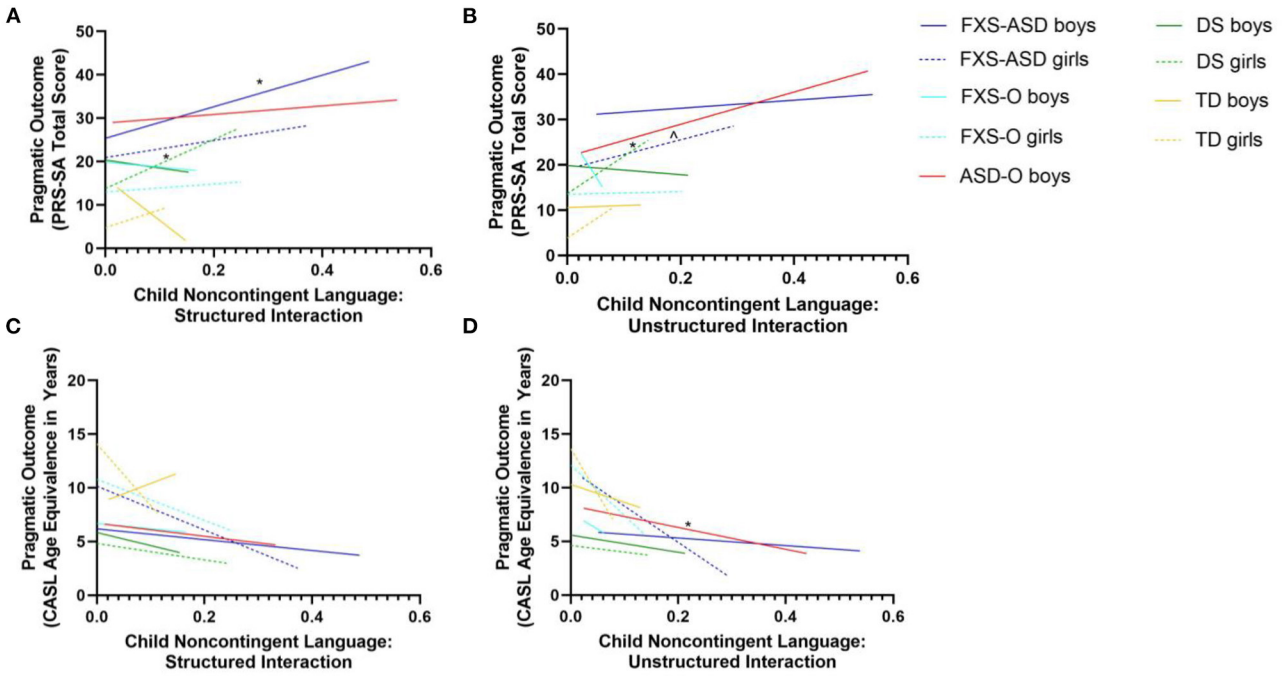

FIGURE 1 | (A–D) Non-contingent language and pragmatic language outcomes. Associations between non-contingent language and pragmatic language outcomes. **(A)** Increased rates of non-contingent language in the structured interactions were significantly associated with poorer pragmatic language outcomes, as measured by the PRS-SA, in FXS-ASD boys and DS girls. **(B)** Increased use of non-contingent language in the unstructured interactions was correlated with poorer pragmatic language outcomes, as measured by the PRS-SA, in the FXS-ASD girls and DS girls. **(C)** There were no significant associations between non-contingent language and pragmatic language outcomes, as measured by the CASL, in any groups. **(D)** Increased rates of non-contingent language in the unstructured interactions was significantly associated with poorer pragmatic language outcomes on the CASL in boys with ASD. FXS-ASD, fragile X syndrome with autism spectrum disorder; FXS-O, FXS only; ASD-O, ASD only; DS, Down syndrome; TD, typical development. ^$p < 0.1$, *$p < 0.05$.

Pragmatic Language Outcomes

Results from longitudinal analyses suggest meaningful parent and child language variables as potential factors related to long-term child pragmatic outcomes, with slightly different patterns emerging across groups. For example, higher rates of non-contingent and perseverative language were associated with greater pragmatic difficulties in boys with FXS-ASD. Not surprisingly, and similar to boys with FXS-ASD, a relationship between increased non-contingent language and poorer pragmatic language outcomes also emerged among boys with ASD-O. This suggests that child non-contingent language during parent-child interactions in both ASD groups predicts greater pragmatic difficulty later in development. These findings highlight an important target for intervention efforts in both ASD groups, and may suggest the ways in which this shared aspect of the pragmatic phenotype in idiopathic and syndromic ASD contributes to developmental outcomes. While the opposite relationship emerged for girls with FXS-ASD, it should be noted that this was of marginal significance. Interestingly, in boys with FXS-O, increased rates of child perseverative and non-responsive language were associated with greater pragmatic deficits 2 years later. Consistent with the literature, this finding suggests that regardless of ASD status, perseveration likely represents an important target in pragmatic language interventions for children with FXS (87), as results indicate it may be related to a broader pattern of pragmatic language difficulties over time. In the context of these findings, clarifying the presence of ASD symptomatology in individuals with FXS has important implications for developing tailored treatment plans aimed at improving pragmatic language outcomes, with areas of meaningful clinical overlap and divergence in the development of treatment goals.

In girls with FXS-O, higher rates of parental non-contingent language during parent-child interactions were associated with greater pragmatic difficulties 2 years later. While not statistically significant, marginal relationships emerged in a similar direction between parental non-responsiveness and perseveration in boys with FXS-ASD. While larger samples and increased power may have yielded a clearer pattern of results, these findings provide an initial indication that parent language style during parent-child interactions may contribute to the child's pragmatic outcomes, even during the school-age years, highlighting an important clinical target that could be addressed in future parent-child intervention studies in FXS.

In contrast, a slightly different pattern emerged in boys with ASD-O, such that increased rates of parent initiations were associated with poorer pragmatic language outcomes on the CASL, a standardized measure of pragmatic language 2 years later. A similar finding was seen among girls with DS. Notably, the content and quality of the parents' initiations in these cases are not entirely clear, although it may be the case that overly frequent parental initiations occurred at the expense of reciprocal parent-child communication. Alternatively, particularly among the ASD-O group, it is also quite possible that parents over-initiated as a way to compensate for their child's non-responsive behaviors, which may thus serve as a mediating factor in this complicated, transactional relationship.

Study Strengths, Limitations, and Clinical and Research Directions

An important contribution of this study was the inclusion of multiple clinical groups included with individuals with FXS. This afforded analysis of pragmatic language profiles that may be unique to FXS, as well as how pragmatic abilities in children with FXS might be influenced by ASD symptoms. Examining language samples in two different contexts, in relationship to parental pragmatic language, and over time offered additional, rich information to further specify pragmatic language abilities and developmental outcomes in FXS. Finally, this study is among the first in to examine the impact of parent-child interactions in school-age children in FXS, ASD, and DS, as the majority of work in this area has focused on toddlers and preschool-age children.

An important limitation of the study is the reliance on the ADOS as the single method of ASD classification. Most individuals with FXS had not been clinically evaluated for ASD previously, and due to time restrictions and participant retention considerations, the ADI-R could only be administered to roughly half the sample, necessitating reliance on the ADOS for group classification [see also (88)]. Future studies should rely on multiple gold-standard assessments, alongside best estimate clinical judgment when possible in providing categorical groupings such as these. In addition, the absence of girls with ASD-O in this study limited conclusions regarding whether patterns found in girls with FXS-ASD extend to idiopathic ASD. Similarly, children with DS who met criteria for ASD based on the ADOS were excluded from the larger study. Specific cognitive and language requirements were also imposed as part of inclusion criteria into the study. This limits the generalizability of findings for this particular group (as well as for participants with FXS). Future studies should include a DS group with and without ASD to better understand the impact of ASD symptomatology on pragmatic language in this group, and whether similar differences exist to what is observed in FXS (i.e., it may be possible that while contingent discourse was a strength in this particular sample, individuals with DS with co-occurring ASD show difficulties in this aspect of language more similar to those with ASD-O and FXS-ASD). Future studies should also examine parent-child interaction styles with larger samples of fathers, to examine potential parent-specific effects that may differ between mothers and fathers (especially among mothers with the *FMR1* premutation). The reduced sample size of the longitudinal data available in this study also limited our ability to examine outcomes for some of the groups included in this study, though nonetheless provided valuable information on potential influences on pragmatic language growth in FXS, ASD, and DS over time, that should be replicated in larger samples. It is also important to note that the number of analyses increased the risk for Type 1 error overall. While reducing Type 2 error was the primary

concern given the novelty of these unique data, the fact that questions were intended to guide next steps in future research, and the difficulties ascertaining a sample such as this, the risk for Type 1 error should be considered in interpreting findings.

Findings suggest several potential clinical implications. First, across idiopathic and syndromic ASD groups, we found that non-contingent language and perseveration represent a core and shared area of deficit. Clinical interventions designed to improve these deficits in ASD can facilitate social communicative skills and social competency more broadly. Many such interventions have been developed specifically for individuals with idiopathic ASD, and it will be important to examine the efficacy of these evidenced-based interventions among individuals with FXS-ASD. Further, the pragmatic impairments documented across these school-age groups could impact the ability to develop peer relationships, and as such, constitute important pragmatic skills that can be targeted in social communication interventions aimed at improving these specific language deficits throughout this age period. It will also be important to continue to provide parents with concrete strategies for how to best adapt their own pragmatic skills in the context of conversational interactions with their child, which may vary over the course of their child's development. This could be incorporated into already existing interventions in which parents serve as "social coaches" [e.g., (89)] for their children. Finally, that children in all groups showing increased pragmatic difficulties during unstructured interactions suggests that clinicians and researchers should incorporate both structured and unstructured assessment contexts in diagnostic evaluations and treatment plans.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of North Carolina at Chapel Hill Institutional Review Board and Northwestern University Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

LB, GM, and ML contributed to conception and design of the study. LB and EL completed all data coding. LB performed the statistical analysis and wrote the first draft of the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Baron-Cohen S. Social and pragmatic deficits in autism: cognitive or affective? *J Autism Dev Disord.* (1988) 18:379–402.
2. Berko-Gleason J, Bernstein-Ratner N. *The Development of Language*. 7th ed. Boston, MA: Pearson (2009).
3. Bruner J. Play, thought, and language. *Peabody J Educ.* (1983) 60:60–9.
4. Bruner J. *Acts of Meaning*. Cambridge, MA; London: Harvard University Press (1990).
5. Grice HP. *Logic and Conversation*. Cambridge, MA: Academic Press (1975).
6. Helland WA, Lundervold AJ, Heimann M, Posserud MB. Stable associations between behavioral problems and language impairments across childhood - the importance of pragmatic language problems. *Res Dev Disabil.* (2014) 35:943–51. doi: 10.1016/j.ridd.2014.02.016
7. Mey J. *Pragmatics: An Introduction*. Oxford: Blackwell (1993).
8. Prutting CA, Kirchner DM. A clinical appraisal of the pragmatic aspects of language. *J Speech Hear Disord.* (1987) 52:105–19.
9. Swineford LB, Thurm A, Baird G, Wetherby AM, Swedo S. Social (pragmatic) communication disorder: a research review of this new DSM-5 diagnostic category. *J Neurodev Disord.* (2014) 6:41. doi: 10.1186/1866-1955-6-41
10. Tomasello M. *Origins of Human Communication*. Cambridge, MA: MIT Press (2008). doi: 10.7551/mitpress/7551.001.0001
11. Ketelaars MP, Cuperus J, Jansonius K, Verhoeven L. Pragmatic language impairment and associated behavioural problems. *Int J Lang Commun Disord.* (2010) 45:204–14. doi: 10.3109/13682820902863090
12. American Psychiatric Association. *Diagnostic and Statistical Manual of Mental Disorders*. 5th ed. American Psychiatric Association (2013). Available online at: http://www.PsychiatryOnline.org (accessed May, 2017).
13. Kaufmann W, Kidd SA, Andrews HF, Budimirovic DB, Esler A, Haas-Givler B, et al. Autism Spectrum Disorder in Fragile X Syndrome: Cooccurring Conditions and Current Treatment. *Pediatrics*. (2017) 139(Suppl. 3):S194–206. doi: 10.1542/peds.2016-1159F
14. Muhle R, Trentacoste SV, Rapin I. The genetics of autism. *Pediatrics.* (2004) 113:e472–86. doi: 10.1542/peds.113.5.e472

15. Schaefer GB, Mendelsohn NJ, Professional P, Guidelines C. Clinical genetics evaluation in identifying the etiology of autism spectrum disorders: 2013 guideline revisions. *Genet Med.* (2013) 15:399–407. doi: 10.1038/gim.2013.32
16. Budimirovic D, Haas-Givler B, Blitz R, Esler A, Kaufmann W, Sudhalter V, et al. *Understanding Autism Spectrum Disorder in Fragile X Syndrome*. Fragile X Clinican & Research Consortium (2014).
17. Martin GE, Bush L, Klusek J, Patel S, Losh M. A multimethod analysis of pragmatic skills in children and adolescents with fragile X syndrome, autism spectrum disorder, and down syndrome. *J Speech Lang Hear Res.* (2018) 61:3023–37. doi: 10.1044/2018_JSLHR-L-18-0008
18. Hale CM, Tager-Flusberg H. Brief report: the relationship between discourse deficits and autism symptomatology. *J Autism Dev Disord.* (2005) 35:519–24. doi: 10.1007/s10803-005-5065-4
19. Tager-Flusberg H, Anderson M. The development of contingent discourse ability in autistic children. *J Child Psychol Psychiatry Allied Disciplines.* (1991) 32:1123–34. doi: 10.1111/j.1469-7610.1991.tb00353.x
20. Losh M, Klusek J, Martin GE, Sideris J, Parlier M, Piven J. Defining genetically meaningful language and personality traits in relatives of individuals with fragile X syndrome and relatives of individuals with autism. *Am J Med Genet B.* (2012) 660–8. doi: 10.1002/ajmg.b.32070
21. Losh M, Martin GE, Klusek J, Hogan-Brown AL, Sideris J. Social communication and theory of mind in boys with autism and fragile x syndrome. *Front Psychol.* (2012) 3:266. doi: 10.3389/fpsyg.2012.00266
22. Klusek J, Martin GE, Losh M. A comparison of pragmatic language in boys with autism and fragile X syndrome. *J Speech Lang Hear Res.* (2014) 57:1692–707. doi: 10.1044/2014_JSLHR-L-13-0064
23. Martin GE, Barstein J, Hornickel J, Matherly S, Durante G, Losh M. Signaling of noncomprehension in communication breakdowns in fragile X syndrome, Down syndrome, and autism spectrum disorder. *J Commun Disord.* (2017) 65:22–34. doi: 10.1016/j.jcomdis.2017.01.003
24. Freeman SF, Kasari C. Characteristics and qualities of the play dates of children with Down syndrome: emerging or true friendships? *Am J Mental Retard.* (2002) 107:16–31. doi: 10.1352/0895-8017(2002)107<0016:CAQOTP>2.0.CO;2
25. Martin GE, Klusek J, Estigarribia B, Roberts JE. Language characteristics of individuals with down syndrome. *Top Lang Disord.* (2009) 29:112–32. doi: 10.1097/TLD.0b013e3181a71fe1
26. Moore DG, Oates JM, Hobson RP, Goodwin J. Cognitive and social factors in the development of infants with Down syndrome. *Downs Syndr Res Pract.* (2002) 8:43–52. doi: 10.3104/reviews.129
27. Abbeduto L, and Chapman RS. (2005). Language development in Down syndrome and fragile X syndrome: current research and implications for theory and practice. In: Fletcher P, Miller JF, editors. *Developmental Theory and Language Disorders*. Trends in Language Acquisition Research (2005). p. 53–72. doi: 10.1075/tilar.4.05abb
28. Martin GE, Losh M, Estigarribia B, Sideris J, Roberts J. Longitudinal profiles of expressive vocabulary, syntax and pragmatic language in boys with fragile X syndrome or Down syndrome [Research Support, NIH, Extramural Research Support, Non-US Gov't]. *Int J Lang Commun Disord.* (2013) 48:432–43. doi: 10.1111/1460-6984.12019
29. Roberts J, Martin GE, Moskowitz L, Harris AA, Foreman J, Nelson L. Discourse skills of boys with fragile X syndrome in comparison to boys with Down syndrome. *J Speech Lang Hear Res.* (2007) 50:475–92. doi: 10.1044/1092-4388(2007/033)
30. Tannock R. Mothers' directiveness in their interactions with their children with and without Down syndrome. *Am J Mental Retard.* (1988) 93:154–65.
31. Abbeduto L, Murphy MM, Kover ST, Giles ND, Karadottir S, Amman A, et al. Signaling noncomprehension of language: a comparison of fragile X syndrome and Down syndrome. *Am J Mental Retard.* (2008) 113:214–30. doi: 10.1352/0895-8017(2008)113[214:SNOLAC]2.0.CO;2
32. Laws G, Bishop D. Pragmatic language impairment and social deficits in Williams syndrome: a comparison with Down's syndrome and specific language impairment. *Int J Lang Commun Disord.* (2004) 39:45–64. doi: 10.1080/13682820310001615797
33. Thurm A, Farmer C, Salzman E, Lord C, Bishop S. State of the field: differentiating intellectual disability from autism spectrum disorder. *Front Psychiatry.* (2019) 10:526. doi: 10.3389/fpsyt.2019.00526
34. Humphrey N, Symes W. Peer interaction patterns among adolescents with autistic spectrum disorders (ASDs) in mainstream school settings. *Autism.* (2011) 15:397–419. doi: 10.1177/1362361310387804
35. Orsmond GI, Kuo HY. The daily lives of adolescents with an autism spectrum disorder: discretionary time use and activity partners. *Autism.* (2011) 15:579–99. doi: 10.1177/1362361310386503
36. Parsons L, Cordier R, Munro N, Joosten A, Speyer R. A systematic review of pragmatic language interventions for children with autism spectrum disorder. *PLoS ONE.* (2017) 12:e0172242. doi: 10.1371/journal.pone.0172242
37. Brown BB, Dietz EL. Informal peer groups in middle childhood and adolescence. In: Rubin K, Bukowski W, Laursen B, editors. *Handbook of Peer Interactions, Relationships, and Groups*. New York, NY: Guilford Publications (2008). p. 654.
38. Haden CA, Haine RA, Fivush R. Developing narrative structure in parent-child reminiscing across the preschool years. *Dev Psychol.* (1997) 33:295–307.
39. Fivush R, Haden CA, Reese E. Elaborating on elaborations: role of maternal reminiscing style in cognitive and socioemotional development. *Child Dev.* (2006) 77:1568–88. doi: 10.1111/j.1467-8624.2006.00960.x
40. Fivush R. The social construction of personal narratives. *Merrill Palmer Quart.* (1991) 37:9–81.
41. Nelson K. Structure and strategy in learning to talk. *Monogr Soc Res Child Dev.* (1973) 38:1–135. doi: 10.2307/1165788
42. Haebig E, McDuffie A, Ellis Weismer S. Brief report: parent verbal responsiveness and language development in toddlers on the autism spectrum. *J Autism Dev Disord.* (2013) 43:2218–27. doi: 10.1007/s10803-013-1763-5
43. Siller M, Sigman M. The behaviors of parents of children with autism predict the subsequent development of their children's communication. *J Autism Dev Disord.* (2002) 32:77–89. doi: 10.1023/A:1014884404276
44. Siller M, Sigman M. Modeling longitudinal change in the language abilities of children with autism: parent behaviors and child characteristics as predictors of change. *Dev Psychol.* (2008) 44:1691–704. doi: 10.1037/a0013771
45. Stern YS, Maltman N, Roberts MY. The influence of maternal pragmatics on the language skills of children with autism. *J Dev Behav Pediatr.* (2017) 38:339–44. doi: 10.1097/DBP.0000000000000445
46. Edmunds SR, Kover ST, Stone WL. The relation between parent verbal responsiveness and child communication in young children with or at risk for autism spectrum disorder: a systematic review and meta-analysis. *Autism Research.* (2019) 12:715–31. doi: 10.1002/aur.2100
47. Warren SF, Brady N, Sterling A, Fleming K, Marquis J. Maternal responsivity predicts language development in young children with fragile X syndrome. *Am J Intellect Dev Disabil.* (2010) 115:54–75. doi: 10.1352/1944-7558-115.1.54
48. Whyte EM, Nelson KE. Trajectories of pragmatic and nonliteral language development in children with autism spectrum disorders. *J Commun Disord.* (2015) 54:2–14. doi: 10.1016/j.jcomdis.2015.01.001
49. Landa R, Folstein SE, Isaacs C. Spontaneous narrative-discourse performance of parents of autistic individuals. *J Speech Hear Res.* (1991) 34:1339–45.
50. Landa R, Piven J, Wzorek MM, Gayle JO, Chase GA, Folstein SE. Social language use in parents of autistic individuals. *Psychol Med.* (1992) 22:245–54.
51. Losh M, Childress D, Lam K, Piven J. Defining key features of the broad autism phenotype: a comparison across parents of multiple- and single-incidence autism families. *Am J Med Genet B.* (2008) 424–33. doi: 10.1002/ajmg.b.30612
52. Losh M, Martin GE, Klusek J, Hogan-Brown AL. Pragmatic Language in autism and fragile X syndrome: genetic and clinical applications. *Perspect Lang Learn Educ.* (2012) 19:48–55. doi: 10.1044/lle19.2.48
53. Piven J, Palmer P, Landa R, Santangelo S, Jacobi D, Childress D. Personality and language characteristics in parents from multiple-incidence autism families. *Am J Med Genet.* (1997) 74:398–411.
54. Maltman N, Guilfoyle J, Nayar K, Martin GE, Winston M, Lau JCY, et al. The phenotypic profile associated with the FMR1 premutation in women: an investigation of clinical-behavioral, social-cognitive, and executive abilities. *Front Psychiatry.* (2021) 12:718485. doi: 10.3389/fpsyt.2021.718485
55. Klusek J, McGrath SE, Abbeduto L, Roberts JE. Pragmatic language features of mothers with the FMR1 premutation are associated with the language outcomes of adolescents and young adults with fragile X syndrome. *J Speech Lang Hear Res.* (2016) 59:49–61. doi: 10.1044/2015_JSLHR-L-15-0102

56. Taylor LJ, Maybery MT, Wray J, Ravine D, Hunt A, Whitehouse AJ. Brief report: do the nature of communication impairments in autism spectrum disorders relate to the broader autism phenotype in parents? *J Autism Dev Disord.* (2013) 43:2984–9. doi: 10.1007/s10803-013-1838-3
57. Murphy MM, Abbeduto L. Gender differences in repetitive language in fragile X syndrome. *J Intellect Disability Res.* (2007) 51(Pt 5):387–400. doi: 10.1111/j.1365-2788.2006.00888.x
58. Losh M, Capps L. Narrative ability in high-functioning children with autism or Asperger's syndrome. *J Autism Dev Disord.* (2003) 33:239–51. doi: 10.1023/a:1024446215446
59. Tamis-LeMonda CS, Bornstein MH, Baumwell L. Maternal responsiveness and children's achievement of language milestones. *Child Dev.* (2001) 72:748–67. doi: 10.1111/1467-8624.00313
60. Lord C, Rutter M, DeLavore PC, Risi S. *Autism Diagnostic Observation Schedule*. Los Angeles, CA: Western Psychological Services (2001).
61. Lord C, Rutter M, Le Couteur A. Autism diagnostic interview-revised: a revised version of a diagnostic interview for caregivers of individuals with possible pervasive developmental disorders. *J Autism Dev Disord.* (1994) 24:659–85.
62. Gotham K, Pickles A, Lord C. Standardizing ADOS scores for a measure of severity in autism spectrum disorders. *J Autism Dev Disord.* (2009) 39:693–705. doi: 10.1007/s10803-008-0674-3
63. Roid GH, Miller LJ. *Leiter International Performance Scale-Revised*. Wood Dale, IL: Stoelting (1997). doi: 10.1037/t05120-000
64. Williams KT. *Expressive Vocabulary Test*. Circle Pines, MN: American Guidance Service (1997).
65. Dunn LM, Dunn DM. *Peabody Picture Vocabulary* Test. 3rd ed. Circle Pines, MN: American Guidance Service (1997).
66. Dunn LM, Dunn DM. *Peabody Picture Vocabulary Test*. 4th ed. Bloomington, MN: Pearson Assessments (2007). doi: 10.1037/t15144-000
67. Brown R. *A First Language: The Early Stages*. Cambridge, MA: Harvard University Press (1973). doi: 10.4159/harvard.9780674732469
68. Miller J, Iglesias A. *Systematic Analysis of Language Transcripts (SALT), 2012 [Computer Software]*. Middleton, WI: SALT Software, LLC (2008).
69. Carrow-Woolfolk E. *CASL: Comprehensive Assessment of Spoken Language*. Circle Pines, MN: American Guidance Services (1999).
70. Lee M, Bush L, Martin GE, Barstein J, Maltman N, Klusek J, et al. A multi-method investigation of pragmatic development in individuals with down syndrome. *Am J Intellect Dev Disabil.* (2017) 122:289–309. doi: 10.1352/1944-7558-122.4.289
71. Landa R. *Pragmatic Rating Scale for School-Age Children*. Baltimore, MD: Kennedy Kreiger Institute (2011).
72. Shrout PE, Fleiss JL. Intraclass correlations: uses in assessing rater reliability. *Psychol Bull.* (1979) 86:420–8. doi: 10.1037/0033-2909.86.2.420
73. Koo TK, Li MY. A guideline of selecting and reporting intraclass correlation coefficients for reliability research. *J Chiropr Med.* (2016) 15:155–63. doi: 10.1016/j.jcm.2016.02.012
74. Reindal L, Naerland T, Weidle B, Lydersen S, Andreassen OA, Sund AM. Structural and pragmatic language impairments in children evaluated for autism spectrum disorder (ASD). *J Autism Dev Disord.* (2021). doi: 10.1007/s10803-020-04853-1. [Epub ahead of print].
75. Lakens D. Calculating and reporting effect sizes to facilitate cumulative science: a practical primer for t-tests and ANOVAs. *Front Psychol.* (2013) 4:863. doi: 10.3389/fpsyg.2013.00863
76. Perneger TV. What's wrong with Bonferroni adjustments. *BMJ.* (1998) 316:1236–8.
77. Budimirovic DB, Schlageter A, Filipovic-Sadic S, Protic DD, Bram E, Mahone EM, et al. A genotype-phenotype study of high-resolution FMR1 nucleic acid and protein analyses in fragile X patients with neurobehavioral assessments. *Brain Sci.* (2020) 10:694. doi: 10.3390/brainsci10100694
78. Koegel RL, Bradshaw JL, Ashbaugh K, Koegel LK. Improving question-asking initiations in young children with autism using pivotal response treatment. *J Autism Dev Disord.* (2014) 44:816–27. doi: 10.1007/s10803-013-1932-6
79. Mundy P, Sigman M, Ungerer J, Sherman T. Defining the social deficits of autism: the contribution of non-verbal communication measures. *J Child Psychol Psychiatry.* (1986) 27:657–69. doi: 10.1111/j.1469-7610.1986.tb00190.x
80. Stone WL, Ousley OY, Yoder PJ, Hogan KL, Hepburn SL. Nonverbal communication in two- and three-year-old children with autism. *J Autism Dev Disord.* (1997) 27:677–96.
81. Wetherby AM, Prutting CA. Profiles of communicative and cognitive-social abilities in autistic children. *J Speech Hear Res.* (1984) 27:364–77.
82. Capps L, Kehres J, Sigman M. Conversational abilities among children with autism and children with developmental delays. *Autism.* (1998) 2:325–44. doi: 10.1177/1362361398024002
83. Loveland KA, Landry SH, Hughes SO, Hall SK, McEvoy RE. Speech acts and the pragmatic deficits of autism. *J Speech Hear Res.* (1988) 31:93–604.
84. Cordeiro L, Ballinger E, Hagerman R, Hessl D. Clinical assessment of DSM-IV anxiety disorders in fragile X syndrome: prevalence and characterization. *J Neurodev Disord.* (2011) 3:57–67. doi: 10.1007/s11689-010-9067-y
85. Wolf-Schein EG, Sudhalter V, Cohen IL, Fisch GS, Hanson D, Pfadt AG, et al. Speech-language and the fragile X syndrome: initial findings. *ASHA*. (1987) 29:35–8.
86. Sudhalter V, Belser RC. Conversational characteristics of children with fragile X syndrome: tangential language. *Am J Ment Retardat AJMR.* (2001) 106:389–400. doi: 10.1352/0895-8017(2001)106<0389:CCOCWF>2.0.CO;2
87. McDuffie A, Machalicek W, Bullard L, Nelson S, Mello M, Tempero-Feigles R, et al. A spoken-language intervention for school-aged boys with fragile X syndrome. *Am J Intellect Dev Disabil.* (2016) 121:236–65. doi: 10.1352/1944-7558-121.3.236
88. Klusek J, Martin GE, Losh M. Consistency between research and clinical diagnoses of autism among boys and girls with fragile X syndrome. *J Intell Disab Res.* (2014) 58:940–52. doi: 10.1111/jir.12121
89. Laugeson E. *PEERS® for Young Adults: Social Skills Training for Adults With Autism Spectrum Disorder and Other Social Challenges*. Routledge (2017).

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Cellular Bioenergetics and AMPK and TORC1 Signalling in Blood Lymphoblasts Are Biomarkers of Clinical Status in FMR1 Premutation Carriers

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Fragile X Associated Tremor/Ataxia Syndrome (FXTAS) is a neurodegenerative disorder affecting carriers of premutation alleles (PM) of the X-linked FMR1 gene, which contain CGG repeat expansions of 55–200 range in a non-coding region. This late-onset disorder is characterised by the presence of tremor/ataxia and cognitive decline, associated with the white matter lesions throughout the brain, especially involving the middle cerebellar peduncles. Nearly half of older male and ∼ 20% of female PM carriers develop FXTAS. While there is evidence for mitochondrial dysfunction in neural and some peripheral tissues from FXTAS patients (though less obvious in the non-FXTAS PM carriers), the results from peripheral blood mononuclear cells (PBMC) are still controversial. Motor, cognitive, and neuropsychiatric impairments were correlated with measures of mitochondrial and non-mitochondrial respiratory activity, AMPK, and TORC1 cellular stress-sensing protein kinases, and CGG repeat size, in a sample of adult FXTAS male and female carriers. Moreover, the levels of these cellular measures, all derived from Epstein- Barr virus (EBV)- transformed and easily accessible blood lymphoblasts, were compared between the FXTAS ($N = 23$) and non-FXTAS ($n = 30$) subgroups, and with baseline data from 33 healthy non-carriers. A significant hyperactivity of cellular bioenergetics components as compared with the baseline data, more marked in the non-FXTAS PMs, was negatively correlated with repeat numbers at the lower end of the CGG-PM distribution. Significant associations of these components with motor impairment measures, including tremor-ataxia and parkinsonism, and neuropsychiatric changes, were prevalent in the FXTAS subgroup. Moreover, a striking elevation of AMPK activity, and a decrease in TORC1 levels, especially in the non-FXTAS carriers, were related to the size of CGG expansion. The bioenergetics changes in blood

lymphoblasts are biomarkers of the clinical status of FMR1 carriers. The relationship between these changes and neurological involvement in the affected carriers suggests that brain bioenergetic alterations are reflected in this peripheral tissue. A possible neuroprotective role of stress sensing kinase, AMPK, in PM carriers, should be addressed in future longitudinal studies. A decreased level of TORC1—the mechanistic target of the rapamycin complex, suggests a possible future approach to therapy in FXTAS.

Keywords: FMR1 premutation, Fragile X-associated tremor/ataxia syndrome (FXTAS), motor scores, SCL-90 scale, lymphoblasts' bioenergetics measures, AMPK, TORC1, cognitive measures

INTRODUCTION

Fragile X premutations, which are small expansions of CGG repeat ranging from 55 to 200 in the non-coding region of the Fragile X Mental Retardation 1 (FMR1) X-linked gene, are associated with the variety of abnormal conditions (1). The most severe premutation-associated disorder affecting carriers of the FMR1 premutation allele is the late onset progressive neurodegenerative condition termed Fragile X Associated Tremor/Ataxia Syndrome (FXTAS), affecting 40−50% of male carriers after the age of 55, and 8–16.5% female carriers in the same age group (2–4). The much lower risk of FXTAS in females than in males may be, at least partly, attributed to the protective role of the normal FMR1 allele on the second X chromosome (5), but the existence of other sex-limited protective factors has recently been postulated (6). The standard diagnostic (core) features of FXTAS require one or more of the following pathological changes: intention tremor; cerebellar ataxia; and white matter disease in the middle cerebellar peduncles (MCP sign) seen on magnetic resonance imaging (MRI) (7, 8); white matter disease in the splenium of the corpus callosum (9) has more recently been considered another core FXTAS feature. Additional changes contributing to the diagnosis include parkinsonism, cognitive decline (executive function and memory deficits) in the later stages of this condition, neuropathy (10, 11), and other MRI findings such as global brain atrophy and white matter disease (9, 12–14), especially in the basis pontis, as well as around the lateral ventricles and deep white matter of cerebral hemispheres. Typical FXTAS neuropathological changes are of widespread ubiquitin-positive intranuclear inclusions abundant in neurones and astrocytes (15), extending to autonomic nervous and neuroendocrine systems and myocardial cells (16–18).

One component of the nuclear inclusions is FMR1 mRNA (19), which has previously been found to be elevated in the blood of premutation carriers as a function of increased CGG repeat number (20). These findings have led to a hypothesised pathogenetic mechanism that involves a toxic gain-of-function of the expanded CGG-repeat mRNA, which arises through the adventitious binding/sequestration by the CGG repeat of one or more proteins, contributing to dysfunction and/or death of the cell (21, 22). An alternative model for FXTAS pathogenesis has been proposed, in which "toxic" peptides are generated by initiating translation at non-AUG codons located upstream of the expanded CGG-repeat element repeat-Associated non-ATG (RAN) translation. This process, which generates a poly-glycine peptide that is toxic to cells and was detected in both the intranuclear inclusions of subjects with FXTAS and in the inclusions of the Dutch premutation CGG-repeat mouse model (23, 24). However, most recent analysis of these inclusions in FXTAS post-mortem brains revealed that they are composed principally of ∼200 proteins, with over half involved in RNA binding and/or protein turnover, whilst the allegedly toxic poly-glycine peptide was found at extremely low levels (25). The abundance of the inclusion-associated ubiquitin -and small ubiquitin-like modifier (SUMO)-based modifiers suggests that the inclusions have been formed as the results of increased protein loads and elevated oxidative stress leading to maladaptive autophagy (25). These and other postulated mechanisms, associated with CGG expansions within the premutation range, and leading to the severe neuropathological changes underlying FXTAS, have been reviewed in (26).

Not all individuals carrying the premutation (PM) alleles exhibit the clinical phenotype of FXTAS: nearly half of male carriers do not develop this condition, but some proportion of these non-FXTAS individuals may manifest either its isolated features such as kinetic tremor or cognitive decline, or have other health problems, such as fibromyalgia, seizures, migraine, anxiety/depression, or hypertension, apparently occurring at higher frequencies than in the general population (27–29). A minority of male PM carriers remain asymptomatic regardless of their age.

As with many other neurodegenerative diseases, mitochondrial hypofunction has been suggested to play a role in the cytopathology of FXTAS. Several authors have investigated the status of mitochondrial expression and function in a range of cultured cells from adult PM individuals, including fibroblasts (30–32), as well as post-mortem brains (30, 33) and choroid plexus (34), reporting reduced expression and function of mitochondrial proteins. Mitochondrial hypofunction has also been encountered in the younger carriers (35).

This hypofunction was also encountered in peripheral blood mononuclear cells (PBMC) from PM carriers (32). A more recent multifaceted study of mitochondrial metabolism provided new details relating to metabolic deficits and mitochondrial dysfunction in this tissue from affected and non-affected female premutation carriers (36). These data obtained from human subjects have been supported by evidence based on neural tissue in Knock-In mice premutation models (33, 37, 38).

However, results from EBV-transformed B- lymphocytes (hereafter termed lymphoblasts) derived from a small sample

of male individuals carrying PM alleles (39, 40) showed that mitochondria in those lymphoblasts are hyperactive, with elevation of all mitochondrial respiratory complex activities. These elevations were much more pronounced in the clinically less affected non-FXTAS PM carriers. It is unclear as to whether these elevations might parallel similar early changes in neural cells, or the features seen in short lived, metabolically quiescent peripheral lymphocytes do not reflect more advanced brain pathological processes.

In order to gain better insight into this phenomenon, here we correlate the measures of mitochondrial and non-mitochondrial respiratory activity with the motor, cognitive, and neuropsychiatric impairments seen in the affected PM carriers, on the one hand, and with the size of the CGG expansion, on the other, in a larger and more diverse sample of male and female premutation carriers. Moreover, we include two of the key proteins involved in cellular energy homeostasis: 5′ adenosine monophosphate-activated protein kinase (AMPK), the major sensor of inadequate cellular energy and other stresses (41), and Target of Rapamycin Complex I (TORC1), which is regulated by signals from AMPK, amino acids and growth factors (42, 43). Mitochondrial biogenesis and activity are regulated both by AMPK and TORC1, so the mitochondrial hyperactivity we previously observed in lymphoblasts from a small sample of PM individuals (40) could occur in response to hyperactivity of AMPK, which was indeed found to be elevated in lymphoblasts from a small sample of PM individuals (40). The present results provide new evidence for the relevance of AMPK and mTORC1 signalling status in blood lymphoblasts to the size of CGG expansion regardless of the clinical status of the PM carriers- as well as for the relationship between mitochondrial hyperactivity in these cells and motor, cognitive, and neuropsychiatric impairments in Fragile X Associated Tremor/Ataxia.

MATERIALS AND METHODS

Sample

There were 28 male and 5 female normal controls, and 38 male and 15 female PM participants. All PM participants were adults. Except for one Asian (Chinese) male, all participants were white Caucasians. The source of all male and the 5 female PM participants was a major research project continuing from 2012 at La Trobe University and supported by the National Institutes of Health, USA. This project's male and female participants were originally recruited through fragile X families' admissions to the Victorian Genetic Counselling Clinic of the Murdoch Institute, or referred from several neurology clinics associated with the University of Melbourne and Monash University; the minority (some residing in the other states) were self-referred by postings in the community through The Australian Fragile X Association. Sixteen PM carrier males from this cohort were already included in our earlier publication, where basic cellular metabolism parameters were correlated with white matter lesion burden (39), and a further 6 males were included in a study of the relationship between AMPK and clinical and genotypic measures (40). Thus, of the 38 PM males, 16 were previously unreported. The other source of the female cohort (10 individuals) was an earlier 2008–2010 project supported by a research grant from the National Health and Medical Research of Australia (NHMRC) to ES and DL. These females, who had originally been ascertained either through their Fragile X Syndrome (FXS) children diagnosed at the Genetic Counselling clinics in the states of Victoria and South Australia, or were identified through cascade testing, were incorporated in an earlier study of progression of motor dysfunction based on a larger sample of female PM carriers (6). All PM participants were originally classified as belonging to the "FXTAS" spectrum ("FXTAS"), asymptomatic ("Unaffected"), and "Other" categories [as in: (40)]; the latter category comprising individuals with isolated features occurring in FXTAS, such as fibromyalgia, dementia, isolated kinetic tremor, mild ataxia, anxiety/depression, autism. However, for the purpose of present analysis, 11 carriers in the "Unaffected" category and 19 carriers in the "Other" category were combined into a non- FXTAS group. The healthy control group included 33 participants (with 5 females) recruited with funding support (to PRF, DL, ES, SJA) from the Michael J Fox Foundation as part of a parallel study on Parkinson's disease (2015–2017). All participants signed informed consent for the present study according to protocols approved by the La Trobe University Human Research Ethics Committee (HEC01-85 and HEC15-058).

Protocols

Neurological and Cognitive Measures

Three motor scales: the Unified Parkinson's Disease Rating Scale Part III-Motor (UPDRS-III) (44); the International Cooperative Ataxia Rating Scale (ICARS) (45); and the Clinical Rating Scale for Tremor (CRST) (46) were administered by two neurologists with experience of these scales.

General cognitive functioning was assessed using Addenbrooke's Cognitive Examination Test (ACE-III) (47). The Similarities and Matrix Reasoning subtests of the Wechsler Adult Intelligence Scale (Third Edition; WAIS-III) (48) provided the measures of verbal and non-verbal reasoning, respectively. WAIS-III Digit Span Backward was employed as measure of working memory (48). Executive functioning was also assessed using The Symbol Digit Modalities Test as a measure of information processing speed (49). The delayed recall and discrimination indices of the Hopkins Verbal Learning Test-Revised (HVLT-R) (50), were employed as measures of delayed recall and recognition memory, respectively.

Psychiatric Pathology Test Scores

The Symptom Checklist 90 Revised (SCL-90-R) (51)—a 90 item self-administered questionnaire, was chosen as it can efficiently provide information on a broad range of relevant psychological symptom clusters. Here we report a summary score providing a measure of overall psychological distress—the Global Severity Index (GSI), as well as two specific symptom domains selected a priori: Depression and Anxiety.

FMR1 Molecular Measures

CGG sizing was conducted in the Laboratory of Dr. Tassone at the MIND Institute, UC Davis. Genomic DNA was isolated from

peripheral blood lymphocytes using standard methods (Purygene Kit; Gentra,Inc., Minneapolis, MN). For Southern blot analysis, 10 micrograms of isolated DNA were digested with EcoRI and NruI. Hybridization was performed using the specific FMR1 genomic dig-labelled StB12.3 probe as previously described (52). Genomic DNA was also amplified by PCR (53).

Lymphoblast Culture

Lymphoblastoid cell lines were created by EBV-mediated transformation of cells from the Peripheral Blood Mononuclear Cell (PBMC) layer at the interface of Ficoll-paque Plus (Sigma-Aldrich) gradients as previously described (54).

Lymphoblasts were cultured in T25 flasks in growth medium (Minimum Essential Medium α (Gibco, Life Technologies), supplemented with 10% foetal Bovine Serum (FBS) and 1% Penicillin/Streptomycin) and were cultured in a humidified 5% CO_2 incubator at 37°C. Cells were seeded to at least 2 × 105 cells/mL, and fed every 3 days either by replacing one third of culture medium with fresh medium, or split in a 1:3 ratio of cell culture to fresh medium. All experiments were conducted within 15 passages of recovery from frozen storage. Confluent cultures were harvested and resuspended in 250 μL aliquots in Recovery™ Cell Culture Freezing Medium (Gibco, Life Technologies) and stored at-−80°C. Frozen cells were recovered by thawing at 37°C and seeded into growth medium. Lymphoblast cultures were harvested for experimentation by centrifugation at 500 × g for 5 min.

Mitochondrial Mass and Membrane Potential

Two mitochondrial dyes, Mitotracker Green and Mitotracker® Red CMXRos (ThermoFisher Scientific), were used to estimate mitochondrial mass and membrane potential, as described by Missailidis et al. (55). Measurements were made in duplicate for each cell line, averaged and normalised within every experiment to the values for our standard selected control cell line (C105).

Seahorse Respirometry

Seahorse respirometry was conducted using the Seahorse XFe24 Extracellular Flux Analyzer and Seahorse XF24 FluxPaks (Agilent Technologies). Oxygen consumption rates (OCR) were measured in lymphoblasts that had been cultured in 6 well culture plates (corning) in growth medium prior to experiments. Experiments were conducted as described previously using 8 × 105 cells/well for each lymphoblast cell line. The basal O_2 consumption rate (basal OCR), the decrease in OCR after oligomycin addition [OCR attributable to adenosine triphosphate (ATP) synthesis], the residual OCR after uncoupling with CCCP and blockade of electron transport with rotenone and antimycin A ("non-mitochondrial" OCR) and the "proton leak" (difference between OCR after oligomycin treatment and the "non-mitochondrial" OCR) in pmol/min/well were determined. The results were averaged over 4 replicate wells per experiment and at least 3 independent experiments per cell line.

AMPK Activity

AMPK assays were performed as described by us previously (54). Lysates were prepared from confluent cell lines (∼25 ml) grown in T75 flasks, harvested, lysed in lysis buffer supplemented with phosphatase inhibitors (50 mM Tris.HCl pH 7.4, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 50 mM NaF, 5 mM sodium pyrophosphate) then snap-frozen in liquid nitrogen. Thawed lysates were cleared by centrifugation at 10,000 ×g for 5 min. Supernatant total protein concentrations were determined with the PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific). To concentrate the AMPK protein, 1 mg of total supernatant protein was immunoprecipitated with rabbit polyclonal anti-AMPKα1 antibody α1-(339–358) (56) bound to equilibrated protein A-agarose beads. The beads were recovered and washed four times by centrifugation before being resuspended in 60 μl wash buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 10% glycerol, 0.1% Tween-20). This was named the AMPK slurry. AMPK activity was assayed over 10 min at 30°C by adding 20 μl of the AMPK slurry to 15 μl buffer (5 mM $MgCl_2$, 50 mM HEPES pH 7.4, 0.1% Tween-20, and 1 mM DTT) containing 100 μM SAMS synthetic peptide (NH_2-HMRSAMSGLHLVKRR-COOH). Reactions were started by adding [γ-32P]-ATP (final concentration 200 μM) and stopped by spotting 21 μl onto P81 ion-exchange chromatography paper (Whatman, GE Healthcare). Liquid scintillation counting (Perkin Elmer) was used to measure the incorporation of 32P into the SAMS peptide. Duplicates were averaged and normalised against the average value from all the control cell lines, in each independent experiment.

TORC1 Activity—Phosphorylation State

TORC1 activity in ME/CFS lymphoblast lysates was measured using a time-resolved fluorescence resonance energy transfer (FRET)based multiwell plate assay based on the phosphorylation state of 4E-BP1, a major TORC1 substrate (Cisbio Bioassays). Lymphoblasts were plated in duplicate wells for each cell line in growth medium at 5 × 104 cells/well in a 384-well plate. Lysis buffer was added to each well as per the manufacturer's instructions and the plate mixed on an orbital shaker for 40 min at RT. Lysates from each sample were then transferred to a white-bottom, white-sided 384 well plate (Corning, New York, USA) including various controls according to the manufacturer's instructions. Freshly prepared antibody mix was then added to each well (anti-4E-BP1 antibody labelled with d2 acceptor, and anti-phospho-4E-BP1 antibody labelled with Eu3+-cryptate donor). The plate was incubated at RT for 2 h and scanned using a Clariostar plate reader (BMG, Ortenberg, Germany) by reading the fluorescence emission at two different wavelengths (665 and 620 nm). The ratio of the FRET signal from anti-phospho-4E-BP1 antibody to the donor fluorescence signal from anti-4E-BP1 antibody was measured.

Reactive Oxygen Species (ROS)

Intracellular ROS levels were measured using the Fluorometric Intracellular ROS Kit (MAK145-1KT, Sigma). Lymphoblasts were seeded in triplicate in 90 μL of Dulbecco's phosphate buffered saline (Sigma) at 1.25 × 105 cells/well into a 96 well black, clear flat bottom plate. Fresh reaction mixture was prepared according to the manufacturer's instructions, 100 μL added to duplicate wells for each cell line and 100ul of PBS added to the remaining well to use for background subtraction.

A cell- free control well containing PBS and reaction mix was also included. The plate was incubated in darkness for 1 h at 37°C with 5% CO_2. The fluorescence was then read on a Clariostar microplate reader (excitation = 520, emission = 605 nm). A control cell line (C105) was included in each experiment to allow internal normalisation to control for between- experiment variation.

ATP Steady State Levels

Steady state ATP levels were measured using luciferase ATP-driven luminescence as per the manufacturer's instructions using the ATP Determination Kit (Molecular Probes) as described previously (54). The signal was normalised against that from a control cell line C105 used in every experiment as an internal control.

Statistical Analyses

Pairwise comparison between median of normal controls (individuals in the normal CGG repeated range) vs. FXTAS, controls vs. PM non-FXTAS and FXTAS vs. non-FXTAS for cellular bioenergetics markers were carried out using the non-parametric Mann-Whitney test. For those variables that were significantly associated with age and/or gender, the analyses were conducted using residuals from regression on age or age and gender. The relationship between each cellular bioenergetics marker (outcome) and CGG repeat size was assessed using robust regression, adjusted for age or age and gender whenever appropriate. Spearman's rank correlation was used to compute pairwise correlations between pairs of cellular bioenergetics markers, separately for controls and FXTAS.

Robust regression was used to assess the relationship between each motor score (outcome) and cellular bioenergetics marker (predictor) in the FXTAS sample, adjusted for age and gender whenever appropriate. The Bonferroni correction was used to adjust for multiple testing. All analyses were conducted using software STATA statistical software (version 16.0, StataCorp, College Station, Tex., USA).

RESULTS

Cellular Respiration and Bioenergetic Status in PM Carriers Compared With Non-carrier Controls

The two genetic groups (33 normal controls and 53 FMR1 PM carriers) bore alleles of the CGG trinucleotide repeat in the FMR1 locus that were either in the normal size range (20–40, control group), or in the premutation range (55–199, PM group). For the purpose of this study, the PM carriers were classified into non-FXTAS (N = 30, including 12 females), and FXTAS (n = 23, including 3 females) subgroups, with individuals in the latter subgroup diagnosed on the basis of the revised clinical criteria (57). However, sample sizes differ for individual traits depending on the proportion of missing data.

Since the main focus of this study has been on the relationships of cellular respiration and signalling measures (such as AMPK and TORC1) in blood lymphoblasts with motor and neuropsychiatric changes in the affected PM carriers, we first evaluated cellular functioning in the FXTAS subgroup, compared with non-FXTAS carriers and healthy controls. These results are summarised in **Figure 1**, for major components of cellular respiratory function (ROS) and cellular signalling (AMPK & TORC1 activities) (1a), and for mitochondrial and non-mitochondrial respiration parameters assessed by a Seahorse Respirometry tool (1b). The results show that the AMPK activity and ATP steady state levels are significantly elevated in both FXTAS and non-FXTAS subgroups compared with controls; while the levels of TORC1 activity and ROS levels are consistently reduced in both carrier subgroups compared with controls.

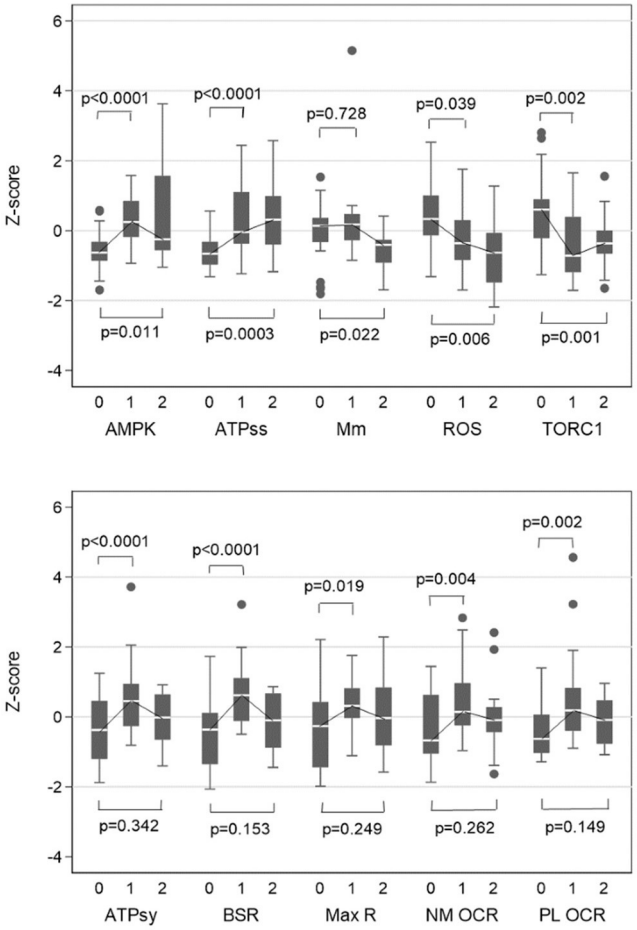
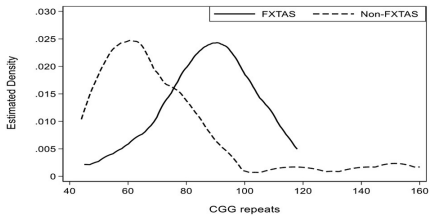


FIGURE 1 | Box plots of Z-scores of biochemical variables for controls vs. Non-FXTAS and controls vs. FXTAS. 0, controls; 1, Non-FXTAS PM; 2, FXTAS; AMPK, Relative AMPK activity; ATPss, ATP steady state level; Mm, Mitochondrial mass; TORC1, TORC1 activity; ATPsy, ATP synthesis; BSR, Basal respiration rate; Max R, Maximum respiration; NM OCR, Non-mitochondrial ATP OCR; PL OCR, Proton Leak OCR. Z-score for BSR and TORC1 were adjusted for age, while ATPsy was adjusted for age and gender. Standardized measures (z-scores) for each variable were computed (for illustrative purpose only) using individual scores summed up over all three subgroups (data) according to the formula: z-score, (data-mean(data))/SD (data).

TABLE 1 | Relationship between biochemical variables (outcome) and CGG repeats, separately for FXTAS and Non-FXTAS assessed by robust regression.

	FXTAS				Non-FXTAS			
	N	**Coef**	**se**	***p****	**N**	**Coef**	**se**	**p***
Relative AMPK activity+	19	1.85	0.96	0.054	28	0.30	0.33	0.364
TORC1 activity+	16	0.10	0.14	0.459	17	0.17	0.33	0.616
Mitochondrial mass+	10	−0.27	0.09	**0.004***	15	0.10	0.03	**0.001***
ROS+	10	0.26	0.82	0.753	15	−0.41	0.10	**<0.001***
Basal respiration rate	17	3.00	1.72	0.081	26	−1.69	0.65	**0.009**
ATP Synthesis	17	1.32	1.36	0.332	26	−1.15	0.49	**0.019**
Non-mitochondrial ATP OCR	17	0.99	0.23	**<0.001***	26	−0.28	0.20	0.169
Proton Leak OCR	17	0.46	0.36	0.195	26	−0.34	0.20	0.077
ATP steady state level+	18	0.84	1.01	0.409	27	−0.88	0.33	**0.008**



Kernel density estimate of distribution of CGG repeat numbers for FXTAS and non-FXTAS subgroups separately.

*Analyses were conducted using robust linear regression; N, sample size; p, p-value; +Estimated regression coefficient (Coef) and standard error (se) were multiplied by 100; *adjusted p-value remained < 0.05 after adjustment for multiple testing using Bonferroni correction method. Bold figures indicate significant relationships prior to adjustment for multiple testing.*

In contrast with the above changes occurring in both carrier subgroups, mitochondrial mass is reduced only in the FXTAS subgroup.

Although the outcome of all Seahorse Respirometry measures are greatly increased in both non-FXTAS and FXTAS subgroups, this increase, relative to controls, is significant only in the former (1b). Direct comparison of these levels between the two subgroups shows, however, that the fall from non-FXTAS to FXTAS is significant only for Basal Respiration Rate ($p = 0.0006$) and ATP Synthesis ($p = 0.0024$).

Consistent with this finding, several elevated Seahorse components—Basal Respiration Rate, ATP-Synthesis and ATP-Steady-State, and reduced level of ROS—all show significant (negative) correlations with CGG repeat size, exclusively in the non-FXTAS subgroup. There is also a positive correlation of CGG with mitochondrial mass in this subgroup. These results contrast with the FXTAS subgroup, where regression coefficients with the same cellular parameters have opposite sign, and the only significant relationship unique to this category occurred with Non-mitochondrial OCR (**Table 1**). Since CGG repeat expansion size is significantly ($p < 0.0001$) higher in FXTAS (89 median) compared with non-FXTAS (65 median) categories (insert in **Table 1**), both the sign and predominance of these correlations in the non-FXTAS group indicate that observed increases in the level of cellular bioenergetics, and decrease in the ROS levels, may be directly linked to repeat sizes within the lower end of CGG distribution for the premutation range. This may explain why, in this study sample, the Seahorse or other related components (except Basal Respiration Rate; $p = 0.035$) were not significantly related to CGG repeat size for the combined (FXTAS and non FXTAS) subgroups (data not shown). The same applies to the AMPK activity and TORC1 levels, though there is a trend, for both these components, to track the CGG repeat size within the < 100 range (see scatterplots in **Figure 2**).

Intercorrelations Between Cellular Markers in PM Carriers and Non-carrier Controls

Exploring intercorrelations between individual cellular bioenergetics components and major cellular energy sensors in PM carriers (especially in the FXTAS subgroup), and non-carrier healthy controls, may be helpful in the interpretation of their relationships with clinical changes. The results in **Table 2** show that the cluster of four significantly intercorrelated major components of cellular bioenergetics—Basal Respiration Rate, ATP Synthesis, Non-mitochondrial OCR and Proton Leak—is identical in both FXTAS and control subgroups. Correlation coefficients are generally lower in FXTAS than in healthy controls, ranging from the highest of 0.925- between Basal respiration rate and ATP Synthesis (in controls), to the lowest of 0.463- between ATP Synthesis and Proton Leak (in FXTAS). Another important similarity between FXTAS and control subgroups is an absence of significant correlations between the above cluster of bioenergetics components, and AMPK, TORC1 and ROS, and between each other within this grouping. The only major difference between FXTAS and controls is the lack of significant relationship of Proton Leak with both Maximum Respiration and Non-mitochondrial OCR, and between the ATP synthesis and non-mitochondrial OCR, in the FXTAS subgroup; compared with the high correlations (0.70, 0.66, 0.63, respectively) seen in the controls (**Table 2**). Notably, in the non-FXTAS subgroup (data not shown), the only apparent difference with the controls is the lack of significant relationship between

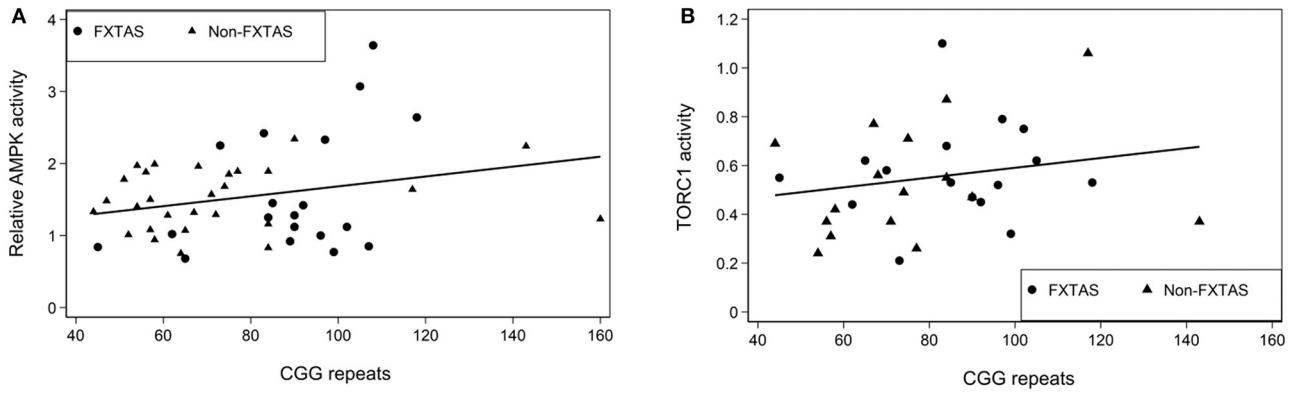


FIGURE 2 | Scatterplots representing the linear relationship between relative AMPK activity **(A)** and TORC1 levels **(B)** in the total sample of PM carriers. Regression of AMPK on CGG: coefficient, 0.0052, $p = 0.181$; regression of TORC1 on CGG: coefficient, 0.0015, $p = 0.404$.

Proton Leak and ATP Synthesis; whereas the latter is, similarly to controls but in contrast with the FXTAS subgroup (shown in **Table 2**), significantly correlated with the non-mitochondrial OCR. Another potentially important difference between healthy control and FXTAS subgroups is the relatively high (0.54) negative correlation between AMPK and mitochondrial mass in FXTAS (although insignificant in a small sample of 10) compared with 0.027 -in a sample of 30 healthy controls (**Table 2**).

Relationships Between Cellular Bioenergetics Markers and Motor Dysfunction, Neuropsychiatric Changes and Cognitive Scores in FXTAS

The purpose of this aspect of our study has been to establish whether the changes in mitochondrial activity and cellular stress signalling encountered in blood lymphoblasts are significantly related to motor, neuropsychiatric and cognitive changes specifically occurring in clinically evident FXTAS. All individual differences between the increases in the three motor scores, and decreases in the cognitive scores included here, were highly significant between FXTAS and the non-FXTAS subgroups ($p < 0.01$). The sole exception was in the SCL90 scores—including GSI total, Depression and Anxiety domains.

The data in **Table 3A** provides a summary of the relationships between key parameters of mitochondrial oxidative phosphorylation and motor impairments assessed by the three motor scales: ICARS, UPDRS, and Clinical Tremor. Notably, there are highly significant correlations between Basal Respiration Rate, Proton Leak and ATP synthesis, and each of the three motor scores (including Gait and Kinetic domains of ICARS). No other significant correlations survived Bonferroni correction. No such relationships have been encountered for the Non-mitochondrial component of Basal respiration, ROS, TORC1, or AMPK activity. It is particularly interesting to note that the score for parkinsonism (UPDRS), which is not a major feature of FXTAS as per standard clinical assessment, shows similar strong relationships with Seahorse major components as the scores for tremor/ataxia represented by ICARS (as illustrated in **Figure 3** by scatterplots showing linear relationships of ATP synthesis with both ICARS total and UPDRS).

Notably, the Non-mitochondrial component, as well as the Basal Respiration Rate, are associated with each of the SCL90 measures—GSI Total, as well as Anxiety and Depression scores—while ATP Synthesis is associated only with the SCL90 Anxiety score. The latter is also (negatively) correlated with ROS (**Table 3B**). The depression domain of SCL90 score ($p = 0.01$) is also highly (negatively) correlated with CGG size (data not shown).

In contrast, no significant relationships were encountered between cellular stress sensing or bioenergetics biomarkers and any cognitive measures. However, ACE-III total, used as a measure of global cognition, and Matrix Reasoning (a measure of non-verbal reasoning), show significant correlations with CGG repeat size in the FXTAS subgroup ($p = 0.008$ and $p < 0.001$, respectively). Matrix Reasoning, and Digit Span Backwards -another measure of executive functioning /working memory—both known to be impaired in otherwise unaffected PM carriers, are also correlated with CGG repeat size in the non-FXTAS subgroup (with p-values of 0.041 and 0.004, respectively). We did not observe any relationship of any other cognitive assays, or motor scores, with either CGG repeat size, or any bioenergetics or cellular stress response markers in this subgroup.

DISCUSSION

Since the first report by Ross-Inta et al. (30), strong evidence has accumulated for decreased mitochondrial bioenergetics in adult FMR1 premutation carriers with or without diagnosable FXTAS. Further data has consistently revealed various aspects of mitochondrial dysfunction in several human tissues-brain as well as peripheral (31, 32, 34, 36, 58, 59). This is supported by evidence based on Knock-in mice premutation models (37, 38). However, evidence from the small number of studies based on blood mononuclear cells (PBMCs) was controversial, showing the presence (36, 60) or the lack of (31) this dysfunction.

TABLE 2 | Spearman's rank correlations among biochemical variables for controls (lower triangle) and FXTAS (upper triangle).

		AMPK	BSR	ATPsy	NM OCR	PL OCR	ATPss	TORC1	ROS	Max R	Mm
AMPK	Corr		0.31	0.39	0.10	−0.10	0.16	0.20	−0.07	0.17	−0.54
	N		19	19	19	19	20	18	9	19	10
	p		0.201	0.103	0.676	0.692	0.510	0.427	0.865	0.479	0.108
BSR	Corr	−0.06		**0.88**+	**0.58**	**0.64**	0.28	−0.16	0.42	**0.75**+	−0.13
	N	30		19	19	19	19	17	9	19	9
	p	0.769		<0.001	0.009	0.003	0.251	0.538	0.265	<0.001	0.732
ATPsy	Corr	−0.08	**0.93+**		0.29	**0.46**	0.26	−0.29	0.33	**0.51**	−0.03
	N	30	33		19	19	19	17	9	19	9
	p	0.664	<0.001		0.226	0.046	0.276	0.251	0.381	0.025	0.932
NM OCR	Corr	0.006	**0.82+**	**0.63**+		0.27	0.20	−0.08	−0.20	**0.72+**	−0.12
	N	30	33	33		19	19	17	9	19	9
	p	0.977	<0.001	<0.001		0.254	0.403	0.754	0.606	<0.001	0.765
PL OCR	Corr	−0.09	**0.72**+	**0.57**+	**0.66**+		0.04	0.24	0.28	0.44	−0.28
	N	29	32	32	32		19	17	9	19	9
	p	0.648	<0.001	0.001	<0.0001		0.870	0.358	0.460	0.063	0.460
ATPss	Corr	−0.17	−0.06	−0.05	0.06	−0.07		0.25	0.36	**0.46**	0.18
	N	30	33	33	33	32		19	10	19	9
	p	0.364	0.749	0.775	0.761	0.714		0.305	0.310	0.049	0.637
TORC1	Corr	−0.19	−0.26	−0.11	−0.35	−0.05	−0.31		−0.15	0.04	−0.57
	N	28	30	30	30	29	30		9	17	7
	p	0.333	0.158	0.579	0.057	0.785	0.095		0.700	0.881	0.180
ROS	Corr	0.12	0.09	0.17	−0.07	−0.27	−0.002	−0.12		0.07	0.19
	N	30	32	32	32	31	32	29		9	8
	p	0.530	0.644	0.357	0.691	0.141	0.993	0.547		0.865	0.651
Max R	Corr	−0.07	**0.89+**	**0.79+**	**0.81**+	**0.70**+	0.05	−0.27	0.11		0.02
	N	30	33	33	33	32	33	30	32		9
	p	0.696	<0.0001	0.0001	<0.001	<0.001	0.772	0.149	0.569		0.966
Mm	Corr	0.027	−0.09	−0.23	0.10	0.06	−0.04	−0.31	**−0.40**	−0.22	
	N	30	33	33	33	32	33	30	32	33	
	p	0.890	0.621	0.208	0.586	0.750	0.815	0.100	0.024	0.228	

N = sample size; p = p-value; +adjusted p-value for Spearman's rank correlation (Corr) remained <0.05 after adjusting for multiple testing using Bonferroni correction. Bold figures indicate significant relationships prior to adjustment for multiple testing.

We previously conducted studies based on immortalised lymphocytes (lymphoblasts) derived from male individuals carrying premutation alleles. We have reviewed and discussed the value of using lymphoblastoid cell lines for this kind of study in our earlier publication (61). In contrast with the majority of those historical findings in other tissues, we reported elevated but functionally normal activities of all mitochondrial respiratory complexes compared with the same data from healthy non-carrier controls (39). Although we used cultured and EBV-transformed lymphoblasts in this study for both premutation and control non-carriers' samples, it is important to note that none of these cultures were allowed to proceed through more than a handful of cell doublings before use in these experiments and they were not used beyond 15 passages.

The above study marked the first time that these cells had been used in correlation with the phenotypic status of PM carriers. The results, based on a small sample of the FXTAS and non-affected carriers combined, showed an elevation of the Basal Respiration Rate and its three components measured by the Seahorse instrument: ATP synthesis; Proton Leak; and Non-mitochondrial OCR. Considering the nature of these findings, an obvious next step was to explore their relevance to FMR1-related clinical phenotypes. Indeed, we found, in the same study, that these increases showed linear association with the extent of white matter lesions, both total and in brain areas suggestive of FMR1 pathology. These data provided the first evidence for the relevance of the bioenergetic changes observed in blood lymphoblastoid cells to the presence and degree of FMR1-associated neurodegeneration.

In the following study (40), based on the same tissue and from the same sample of PM male carriers, we reported heightened cellular stress responses, as manifested by an increase in AMPK kinase activity in both FXTAS and non-FXTAS carrier subgroups, analysed separately. Notably, this increase was highly significant in the non-FXTAS carriers but was less evident in the FXTAS group. Furthermore, this activity showed significant

TABLE 3A | Relationships between motor scores and cellular stress sensing and bioenergetics measures in FXTAS sample.

	Relative AMPK activity				TORC1 activity				Mitochondrial mass			
	N	Coef	se	*p*	N	Coef	se	p	N	Coef	se	*p*
ICARS total	21	−0.20	2.86	0.945	18	3.09	12.4	0.803	9	−5.28	22.6	0.815
ICARS gait+	21	0.23	0.93	0.801	18	−0.65	4.40	0.883	9	−6.97	5.49	0.204
ICARS kinetic	21	0.21	1.59	0.897	18	3.11	5.81	0.592	9	−11.1	20.0	0.579
UPDRS+	19	0.87	2.55	0.733	17	−9.77	11.5	0.394	8	−21.8	26.8	0.416
TREMOR score	21	−1.56	3.34	0.642	18	−10.1	13.7	0.462	9	−21.1	48.2	0.662
	ROS				**Basal respiration rate+**				**ATP Synthesis+**			
ICARS total	9	−11.6	10.6	0.273	18	6.45	1.72	**<0.001***	18	7.59	2.63	**0.004***
ICARS gait+	9	−2.20	2.25	0.328	18	1.92	0.44	**<0.001***	18	2.73	0.51	**<0.001***
ICARS kinetic	9	−3.48	3.78	0.358	18	3.59	0.86	**<0.001***	18	4.05	1.69	**0.016**
UPDRS+	8	−18.8	9.81	0.114	17	6.54	1.41	**<0.001***	17	9.81	1.58	**<0.001***
TREMOR score	9	−4.89	8.03	0.542	18	8.42	3.24	**0.010***	18	7.44	3.09	**0.016**
	Non-mitochondrial ATP OCR+				**Proton Leak OCR+**				**ATP steady state level**			
ICARS total	18	18.7	14.9	0.211	18	33.5	7.44	**< 0.001***	19	4.02	4.88	0.409
ICARS Gait+	18	5.57	3.43	0.104	18	6.88	2.48	**0.006***	19	1.84	0.84	**0.029**
ICARS kinetic	18	8.89	6.71	0.185	18	18.0	4.08	**<0.001***	19	1.41	2.74	0.608
UPDRS+	17	8.07	7.12	0.257	17	26.9	6.66	**<0.001***	18	0.45	3.71	0.902
TREMOR score	18	24.2	16.8	0.151	18	51.5	16.6	**0.002***	19	3.05	5.81	0.600

*N = sample size; p = p-value; +Estimated regression coefficients (Coef) and standard errors (se) were multiplied by 100; +adjusted for age and gender if appropriate; *adjusted p-value remained <0.05 after adjustment for multiple testing using Bonferroni correction method. Bold figures indicate significant relationships prior to adjustment for multiple testing. Motor scale scores are the outcome, and cellular measures are predictors in robust regression analysis.*

(negative) correlations with the ICARS ataxia score, and with the extent of total- as well as supratentorial- white matter lesions, across the FXTAS and non-FXTAS samples combined. These results demonstrated that peripheral cellular stress responses and signalling have, as was the case with cellular bioenergetics components, meaningful links with pathological processes in the brain of male PM carriers.

In order to obtain clearer insight into the mechanisms behind these novel, but still fragmentary, findings in small samples, in the current study we expanded the scope of clinical measures, including all three motor scores, and several cognitive and neuropsychiatric scores, to explore the relevance of the thus-defined phenotype to the two major aspects of cellular pathology: cellular bioenergetics; and cellular stress responses. We used cultured lymphoblasts from a larger sample of carriers. Although we strongly emphasised the concept of a continuum of clinical and neuropathological changes in all PM carriers (2, 6, 62), here we distinguish between these two subgroups (FXTAS and non-FXTAS), with the intention of exploring the trajectory of cellular changes evolving from non- FXTAS to diagnosable FXTAS-as indicative of the potentially much more informative longitudinal approach.

The major finding from the primary comparative analysis, where we tracked the levels of major cellular bioenergetic measures from healthy controls across non-FXTAS to FXTAS subgroups, was a highly significant elevation of all the Seahorse respirometry (bioenergetics) components compared with healthy controls in the non-FXTAS subgroup, and a subsequent fall, in the FXTAS subgroup, to levels closer to, and not significantly elevated above, those of the control subgroup. It is possible however that the FXTAS-control differences are still present, and that the lack of significance is the consequence of the smallness of our sample. All the four major components showing consistent elevation are normally highly intercorrelated, as confirmed by our data from the normal control sample. These relationships were largely mirrored in the FXTAS subgroup, with the exception of proton leak, which was less strongly correlated with the remaining major bioenergetics components in FXTAS compared with healthy controls. These findings are clearly in contrast to the finding of mitochondrial dysfunction in blood lymphocytes from PM females (36), although not with the absence of dysfunction in the same cells from FXTAS males and females combined, as reported by (31).

Our results also show that smaller CGG repeat sizes within the PM range have the most prominent effect on these bioenergetics changes. Thus, the majority of significant correlations between these changes and CGG repeat size occurs in the non-FXTAS subgroup, where CGG size peaks at ∼60 repeats; whereas in the FXTAS subgroup it peaks at ∼90 repeats. The negative correlations between the elevation of these components and the CGG repeat size further strengthen the argument that the overstimulating effect on mitochondrial respiration rate is linked to the lowest end of CGG distribution within the premutation range. In contrast, the increasing CGG repeat numbers at the

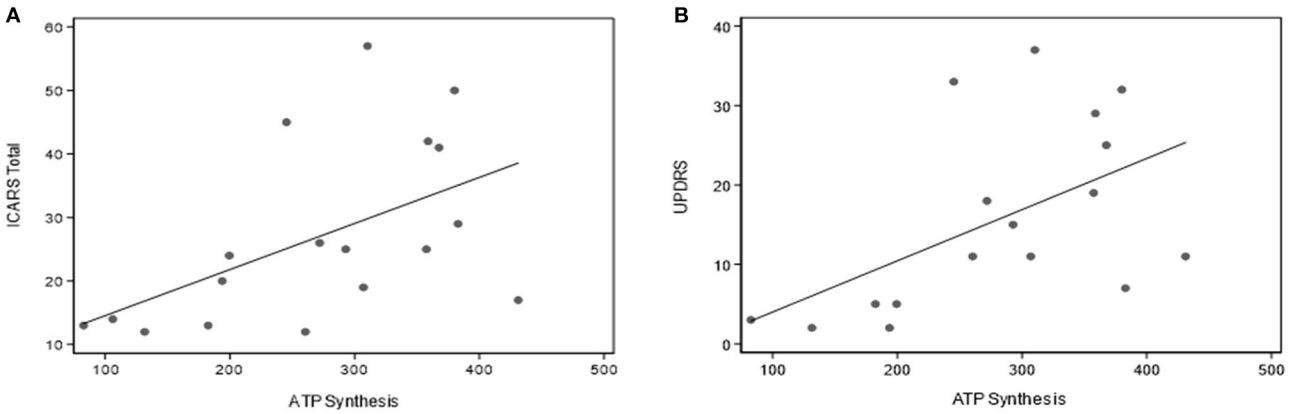


FIGURE 3 | Scatterplots representing the linear relationships between ATP synthesis and ICARS **(A)** and UPDRS **(B)** scores in the total sample of PM carriers.

TABLE 3B | Relationships between SCL90 scores and cellular stress sensing and bioenergetics measures in FXTAS sample.

	Relative AMPK activity				TORC1 activity				Mitochondrial mass			
	N	**Coef**	**se**	***p***	**N**	**Coef**	**se**	***p***	**N**	**Coef**	**se**	***p***
SCL90 depression T score	12	1.27	3.60	0.725	10	−9.48	28.3	0.738	5	−15.0	47.3	0.751
SCl90 anxiety T score	12	1.26	3.85	0.743	10	6.30	37.1	0.865	5	−17.9	14.9	0.229
SCL90 GSI T score	12	0.88	2.09	0.675	10	2.18	13.3	0.870	5	−23.7	59.3	0.690
	ROS				**Basal respiration rate+**				**ATP Synthesis+**			
SCL90 depression T score	5	−18.6	20.0	0.352	11	6.81	1.66	**<0.001***	11	4.47	3.46	0.197
SCL90 anxiety T score	5	−41.8	11.6	**< 0.001***	11	4.41	0.67	**<0.001***	11	5.44	0.84	**<0.001***
SCL90 GSI T score	5	−21.8	14.6	0.134	11	4.62	0.99	**<0.001***	11	3.26	2.92	0.264
	Non-mitochondrial ATP OCR+				**Proton Leak OCR+**				**ATP steady state level**			
SCL90 depression T score	11	28.7	5.03	**<0.001***	11	20.4	13.2	0.121	12	3.68	5.15	0.475
SCL90 anxiety T score	11	11.0	3.09	**< 0.001***	11	17.7	9.58	0.065	12	1.56	4.28	0.715
SCL90 GSI T score	11	15.6	1.81	**<0.001***	11	16.8	9.15	0.066	12	1.79	3.09	0.563

*N = sample size; p = p-value; +Estimated regression coefficient (Coef) and standard error (se) were multiple by 100. *p-value remained <0.05 after adjustment for multiple testing using Bonferroni correction. Bold figures indicate significant relationships prior to adjustment for multiple testing. SCL90 scale scores (not adjusted for age or gender) are the outcome, and cellular measures are predictors in robust regression analysis.*

higher end of the distribution, (corresponding to the FXTAS subgroup), showed significant correlation with a decrease in the mitochondrial mass, as well as with an increase in non-mitochondrial oxygen consumption.

These data provide confirmatory evidence for an absence of detectable mitochondrial dysfunction in blood lymphoblasts from PM carriers without diagnosable FXTAS, which is consistent with the unchanged mitochondrial mass compared with controls. However, the obvious drop in the elevated cellular bioenergetics relative to the non-FXTAS subgroup, combined with a decrease of mitochondrial mass proportional to the increasing CGG repeat size may suggest a progression towards such dysfunction in the FXTAS subgroup. This is apparently consistent with the lack of significant relationship between non-mitochondrial OCR measures and ATP synthesis rates—exclusively in the FXTAS subgroup, which indicates that this synthesis may not be regulated homeostatically in the affected individuals.

Possible mechanisms behind this observed trajectory of Seahorse bioenergetics measures were discussed in our earlier reports (39, 54), but the more fundamental interpretation of these findings, which clearly contradict the evidence of dysfunction in other tissues from PM carriers, is still open to speculation. The most likely explanation is that the observed increase in cellular bioenergetics represents an early stage of cellular pathology in the form of an elevated response to cellular stress, linked to a small CCG repeat expansion. This mitochondrial hyperactivity may have a damaging effect on mitochondrial function, subsequently leading to its decline (with loss of mitochondrial mass) in the later stages of this process. However, the mitochondrial dysfunction stage may not be fully reflected in the lymphoblastoid cells because of the latter's rapid turnover, in contrast with other

tissues such as neurones or fibroblasts (40, 54). More specifically, hyperactivity observed here may be related to the employment of B cells that are selectively immortalised by EBV transformation. Since these cells are involved in the adaptive immune system, they are expected to respond vigorously to the inflammatory component associated with neurodegenerative processes. This well-established association is exemplified by the report by Martinez Cardeno et al. (63) of an increased number and elevated activation state of microglial cells in about half (7 of 13) of their post-mortem FXTAS brains, indicating a neuroinflammatory state. Along the same lines, lymphocyte recruitment, activation and infiltration of the brain tissue has been reported in other neurodegenerative conditions, such as Parkinson's disease and amyotrophic lateral sclerosis (64).

Further insight into this dilemma may be provided by our parallel results from the current study, concerning cellular stress response and signalling in the two PM subgroups, FXTAS and non-FXTAS, represented by the dynamics of the AMPK-mTORC1 pathway. AMPK is a major factor sensing and controlling the level of cellular bioenergetics, mainly through enhancing ATP synthesis, switching off ATP -consuming anabolic pathways and enhancing mitochondrial biogenesis and activity, and autophagy [see: Hardie (65) for extensive review]. There has been strong evidence for multiple potential sources of neuronal stress linked to the elevation of the expanded FMR1 mRNA levels, which leads to corruption/sequestration of specific proteins (26). This phenomenon, combined with transcriptional induction of several stress response genes, was also observed in peripheral fibroblasts from affected (66, 67), as well unaffected (32) human PM carriers.

An increase in AMPK activity across the whole (premutation) range of repeat sizes observed here in cultured lymphoblasts from PM carriers, may be the result of those cellular stresses. The elevation of AMPK activity is typically regarded as adaptive, such that the prominent increase in this activity in the non-FXTAS subgroup was interpreted as being protective against cellular damage leading to FXTAS (39). Although this interpretation was mainly intuitive and based on our genotype-phenotype relationships results, a possible mechanism underlying its protective role in this context might be suggested by findings relevant to histone acetylation. Notably, AMPK has been reported as playing a significant role in the regulation of histone acetylation/deacetylation- as well as being regulated itself by acetylation [i.e., Vancura et al. (68)]. This report may be linked to the earlier data based on both Drosophila premutation model and lymphoblast cell lines derived from two premutation patients with probable FXTAS, showing that histone deacetylases suppress CGG repeat-induced neurodegeneration in FXTAS *via* transcriptional silencing (69). However, the effect of the double-edged sword of elevation of AMPK activity in cellular bioenergetics has also been reported in: (70–72). Since the observed decrease of AMPK activity in the FXTAS subgroup relative to its increase in the non-FXTAS subgroup was only slight and insignificant, our data does not provide adequate information to contemplate this option, which should be addressed in future follow-up studies based on larger samples.

Although the general view holds that there is close signalling interplay between mammalian Target of Rapamycin Complex I (mTORC1) and AMPK, especially in maintaining a balance between the level of anabolic and catabolic processes to preserve cellular homeostasis (73), our findings failed to show significant correlation between these two factors. This result may be partially due to the low statistical power of small samples, especially considering that the role of AMPK and TORC1 in the regulation of homeostasis dynamics may involve separate pathways, with both independently linked to CGG repeat expansions within the PM range. A complexity and multitude of factors affecting TORC1 modulation, and the protective effect of its inhibition, have been discussed earlier (73–75). By the same token, the lack of significant correlations between the levels of these two stress-sensing proteins and the clinical, as well as bioenergetics, measures of FXTAS in our data are not unexpected, considering that their primary controlling role is in cellular homeostasis, and thus their effect on the phenotype may be indirect. Nevertheless, our results clearly demonstrate that the levels of activity of both AMPK and mTORC1 in blood lymphoblasts from FMR1 premutation carriers are significantly related to, and are thus biomarkers of, clinical and genetic status of the carriers. Our comparative results demonstrated an inverse pattern of TORC1 to that of AMPK activities, by showing a highly significant decrease in non-FXTAS relative to healthy controls, and only a slight trend upward in the FXTAS-compared with the non-FXTAS subgroup. However, both AMPK and TORC1 showed a trend towards tracking the CGG repeat size in these two subgroups combined, though, in our data, the respective associations were not statistically significant. More detailed interpretation of the AMPK-TORC1 interrelationship and its relevance to the increasing CGG repeat expansion in the total sample of PM carriers have been given in our earlier publication (76). While the mechanism by which premutation alleles inhibit TORC1 activity is unknown, there are several possibilities including gain of function RNA toxicity (associated with RNA-mediated protein sequestration by elevated FMR1 mRNA levels) and polypeptide toxicity (from accumulation of abnormal, toxic polyG- or polyA-containing RAN translation products). Both of these processes could result in dysregulation of the AMPK-TORC1 signalling axis which regulates mitochondrial biogenesis and activity in response to diverse cellular stresses.

Notably, the decreased level of TORC1 found in our non-FXTAS PM subgroup is consistent with earlier results from the "90R" (premutation) mouse model (77). This showed a protective effect resulting from TORC1's inhibition through increased autophagy, and thus elimination of diseased cells. The recent analysis of the contents of intranuclear inclusions in human FXTAS neurons and astrocytes provided new evidence that elevated oxidative stress and increased loads of protein aggregates lead to these inclusions' formation through impaired autophagy (25). This may implicate mTORC1 as a potential treatment target. Indeed, a beneficial effect of rapamycin (which acts as an allosteric inhibitor of mTORC1) in alleviating non-motor symptoms of parkinsonism, has been reported in a mouse model of non-FMR1 related parkinsonism (78).

The present study is the first to provide evidence for the relationship between the severity of FXTAS neurological phenotype and cellular bioenergetics markers in blood lymphoblasts. These relationships were also significant in the combined non-FXTAS and FXTAS subgroups, but this aspect of analysis was focused on the subgroup meeting the FXTAS diagnostic criteria. This is because, although the non-Fragile X subgroup was not entirely asymptomatic, isolated symptoms occurring in these carriers may not have necessarily been relevant to the FXTAS phenotype, especially in the absence of overt (and age—unrelated) white matter changes in the brain.

All three key parameters of mitochondrial oxidative phosphorylation were highly correlated with each of the three motor scale scores: ICARS, Clinical tremor and UPDRS, in the FXTAS sample. That these correlations also involve the latter scale is somewhat unexpected but noteworthy, since, unlike tremor and ataxia, parkinsonism is not listed as a major feature of FXTAS. This particular result is of considerable interest, considering our earlier finding of a strong relationship between UPDRS score and CGG repeat size in FXTAS (6); and, more recently, new evidence for an elevated risk of parkinsonism in a sibship carrying the premutation/grey zone alleles and affected with kinetic tremor (79). It appears, therefore, that there is more overlap than previously thought between pathological mechanisms underlying both FXTAS and Parkinson's disease such that the former is often misdiagnosed as the latter.

Notably, the Basal Respiration Rate, ATP synthesis and Non-mitochondrial bioenergetics components are also highly correlated with the SCL90 GSI total, and /or Anxiety and Depression domains. This finding is not unexpected considering that neuropsychiatric features have been a major issue in both FXTAS and non-FXTAS carriers, especially females (6, 29, 80–83). Consistent with the extent of these problems across carrier categories, in this study we found significant correlations of these neuropsychiatric features with bioenergetic changes in the FXTAS subgroup, as well as in the combined (FXTAS and non-FXTAS) sample of PM carriers. This result calls for more attention to these well-documented and prevalent neuropsychiatric problems; since they occur both in obviously affected and non-affected PM carriers, the need for early intervention is emphasised, following our earlier recommendations for the female carriers (6). Moreover, both measures of executive functioning (Matrix Reasoning and Digit Span Backward), which are known to be affected early in the non-FXTAS carriers, are significantly correlated with the elevated bioenergetics and cellular stress response components in this subgroup. This suggests the relevance of the hyperactive energy metabolism observed in cultured lymphoblasts to early pathological processes in brain tissue underpinning those specific early clinical manifestations.

The absence of significant relationships between cellular stress sensing or bioenergetics biomarkers and any cognitive scores in the FXTAS subgroup is somewhat unexpected, with only two measures (ACE-III for global cognition, and Matrix Reasoning) being highly correlated with CGG repeat size in the same subgroup. The general absence of these relationships is in contrast with the earlier finding of significant correlation between bioenergetic markers and general cognitive measures, as well as selected measures of executive function (36). However, direct comparison cannot be drawn between the outcome of the two studies, since the earlier one was based on a different cell type (PBMCs), different measures of clinical and cognitive phenotype, and an overall different statistical approach. Apart from the small size of our samples, rigorous age adjustment, and correction for multiple testings, the reason for an absence of detectable relationships of cognitive impairments with our cellular biomarkers could be that cognitive decline is not a major or early feature of FXTAS. If indeed this decline relates to the later stages of the disease process, the relevance of this decline to the early changes represented in the short-lived lymphoblastoid cells (54) may not have been recorded. Clearly, further studies using a longitudinal model and a broader range of potential bioenergetics markers are required to fully address this issue.

In conclusion, one of the major findings from our study was the demonstration that the changes in bioenergetics and stress signalling occurring in cultured, EBV transformed lymphoblasts may be reliable biomarkers of motor and non-motor (especially neuropsychiatric) changes in PM carriers. A corollary of this would be that the dynamics of cellular changes in the transformed lymphoblasts should reflect the pathological processes in the brains of these carriers. An additional practical outcome of this result is that lymphoblastoid cell lines are a readily accessible and enduring cell type available from study participants.

Another major, though still not fully explained, finding is of hyperactivity of cellular bioenergetics components, especially in non-FXTAS PM carriers, compared with the lymphoblasts from non-carrier healthy controls. A particularly important aspect of these results is that the increased level of this hyperactivity in PM carriers is related to the CGG repeat size, but that this relationship is most apparent at the lower end of the CGG distribution.

Finally, the observed dynamics of the cellular stress-sensing protein kinases—AMPK and TORC1—raise an important issue of their possible role in protection against neural damage in FXTAS. These preliminary findings may guide future experimental work to establish the role of these energy sensors and metabolism-controlling enzymes, both being related to CGG repeat size, and both being potential targets for protective measures (such as rapamycin or metformin).

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by La Trobe University Human Research Ethics Committee (HEC01-85 and HEC15-058). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DL: conception, organization, and partial execution of research project, neurological assessments and motor scales scoring, review of statistical analysis, and co-writing (with ES) a manuscript. BK: overseeing and coordinating stress response testing, interpretation of the tests result, contribution to study design, and reviewing and editing the final manuscript. MB: statistical analysis, contribution to study design, and to final editing of manuscript. PF: overseeing and coordinating cellular bioenergetics and stress response testing, interpretation of the test results, contribution to study design, and writing and editing the final manuscript. CA and OS: conducting cellular bioenergetics assays, interpretation of the results and entering and processing the data in the database file, and review of the manuscript. KN: conducting AMPK activity assays, interpretation of the results, review, and critique of the manuscript. AA: contribution to cognitive testing and scoring, creating study database, entering, and processing the data, contribution to statistical analysis, contribution to review, and final editing of manuscript. FT: conduct and interpretation of genetic molecular assays, review, and critique of manuscript. SA: coordination and conduction of cellular bioenergetics assays, interpretation of the tests result, contribution to study design, and writing and editing the final manuscript. ES: conception and partial execution of research project, neurological assessments and motor scales scoring, neuropsychological assessments or supervision of assessments, and co- writing (with DL) of manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Hagerman PJ, Hagerman RJ. The fragile-X premutation: a maturing perspective. *Am J Hum Genet.* (2004) 74:805–16. doi: 10.1086/386296
2. Loesch D, Hagerman R. Unstable mutations in the FMR1 gene and the phenotypes. *Adv Exp Med Biol.* (2012) 769:78–114. doi: 10.1007/978-1-4614-5434-2_6
3. Rodriguez-Revenga L, Madrigal I, Pagonabarraga J, Xunclà M, Badenas C, Kulisevsky J, et al. Penetrance of FMR1 premutation associated pathologies in fragile X syndrome families. *Eur J Hum Genet.* (2009) 17:1359–62. doi: 10.1038/ejhg.2009.51
4. Hagerman R, Hagerman P. Advances in clinical and molecular understanding of the FMR1 premutation and fragile X-associated tremor/ataxia syndrome. *Lancet Neurol.* (2013) 12:786–98. doi: 10.1016/S1474-4422(13)70125-X
5. Jacquemont S, Hagerman RJ, Leehey MA, Hall DA, Levine RA, Brunberg JA, et al. Penetrance of the fragile X-associated tremor/ataxia syndrome in a premutation carrier population. *JAMA.* (2004) 291:460–9. doi: 10.1001/jama.291.4.460
6. Loesch DZ, Tassone F, Atkinson A, Stimpson P, Trost N, Pountney DL, et al. Differential progression of motor dysfunction between male and female fragile x premutation carriers reveals novel aspects of sex-specific neural involvement. *Front Mol Biosci.* (2021) 7:246. doi: 10.3389/fmolb.2020.577246
7. Hagerman RJ, Hagerman P. Fragile X-associated tremor/ataxia syndrome-features, mechanisms and management. *Nat Rev Neurol.* (2016) 12:403–12. doi: 10.1038/nrneurol.2016.82
8. Brunberg JA, Jacquemont S, Hagerman RJ, Berry-Kravis EM, Grigsby J, Leehey MA, et al. Fragile X premutation carriers: characteristic MR imaging findings of adult male patients with progressive cerebellar and cognitive dysfunction. *AJNR Am J Neuroradiol.* (2002) 23:1757–66.
9. Hermanson M, Jhaveri M, Stebbins G, Dunn E, Merkitch D, Berry-Kravis E, et al. The splenium of the corpus callosum sign in fragile x associated tremor ataxia syndrome (FXTAS) (P2.125). *Neurology.* (2015) 84(14 Supplement):P2.125.
10. Apartis E, Blancher A, Meissner WG, Guyant-Maréchal L, Maltête D, De Broucker T, et al. FXTAS: new insights and the need for revised diagnostic criteria. *Neurology.* (2012) 79:1898–907. doi: 10.1212/WNL.0b013e318271f7ff
11. Soontarapornchai K, Maselli R, Fenton-Farrell G, Tassone F, Hagerman PJ, Hessl D, et al. Abnormal nerve conduction features in fragile X premutation carriers. *Arch Neurol.* (2008) 65:495–8. doi: 10.1001/archneur.65.4.495
12. Wheeler AC, Bailey DB. Jr., Berry-Kravis E, Greenberg J, Losh M, Mailick M, et al. Associated features in females with an FMR1 premutation. *J Neurodev Disord.* (2014) 6:30. doi: 10.1186/1866-1955-6-30
13. Adams JS, Adams PE, Nguyen D, Brunberg JA, Tassone F, Zhang W, et al. Volumetric brain changes in females with fragile X-associated tremor/ataxia syndrome (FXTAS). *Neurology.* (2007) 69:851–9. doi: 10.1212/01.wnl.0000269781.10417.7b
14. Loesch DZ, Kotschet K, Trost N, Greco CM, Kinsella G, Slater HR, et al. White matter changes in basis pontis in small expansion FMR1 allele carriers with parkinsonism. *Am J Med Genet B Neuropsychiatr Genet.* (2011) 156:502–6. doi: 10.1002/ajmg.b.31189
15. Greco CM, Hagerman RJ, Tassone F, Chudley AE, Del Bigio MR, Jacquemont S, et al. Neuronal intranuclear inclusions in a new cerebellar tremor/ataxia syndrome among fragile X carriers. *Brain.* (2002) 125(Pt 8):1760–71. doi: 10.1093/brain/awf184
16. Louis E, Moskowitz C, Friez M, Amaya M, Vonsattel JPG. Parkinsonism, dysautonomia, and intranuclear inclusions in a fragile X carrier: a clinical–pathological study. *Mov Disord.* (2006) 21:420–5. doi: 10.1002/mds.20753
17. Greco CM, Soontrapornchai K, Wirojanan J, Gould JE, Hagerman PJ, Hagerman RJ. Testicular and pituitary inclusion formation in fragile X associated tremor/ataxia syndrome. *J Urol.* (2007) 177:1434–7. doi: 10.1016/j.juro.2006.11.097
18. Hunsaker MR, Greco CM, Spath MA, Smits AP, Navarro CS, Tassone F, et al. Widespread non-central nervous system organ pathology in fragile X premutation carriers with fragile X-associated tremor/ataxia syndrome and CGG knock-in mice. *Acta Neuropathol.* (2011) 122:467–79. doi: 10.1007/s00401-011-0860-9
19. Tassone F, Hagerman RJ, Garcia-Arocena D, Khandjian EW, Greco CM, Hagerman PJ. Intranuclear inclusions in neural cells with premutation alleles in fragile X associated tremor/ataxia syndrome. *J Med Genet.* (2004) 41:e43. doi: 10.1136/jmg.2003.012518
20. Tassone F, Hagerman RJ, Taylor AK, Gane LW, Godfrey TE, Hagerman PJ. Elevated levels of FMR1 mRNA in carrier males: a new mechanism of involvement in the fragile-X syndrome. *Am J Hum Genet.* (2000) 66:6–15. doi: 10.1086/302720
21. Jin P, Zarnescu DC, Zhang F, Pearson CE, Lucchesi JC, Moses K, et al. RNA-mediated neurodegeneration caused by the fragile X premutation rCGG repeats in Drosophila. *Neuron.* (2003) 39:739–47. doi: 10.1016/S0896-6273(03)00533-6

22. Polussa J, Schneider A, Hagerman R. Molecular advances leading to treatment implications for fragile x premutation carriers. *Brain Disord Ther.* (2014) 3:19. doi: 10.4172/2168-975X.1000119
23. Todd PK, Oh SY, Krans A, He F, Sellier C, Frazer M, et al. CGG repeat-associated translation mediates neurodegeneration in fragile X tremor ataxia syndrome. *Neuron.* (2013) 78:440–55. doi: 10.1016/j.neuron.2013.03.026
24. Boivin M, Willemsen R, Hukema RK, Sellier C. Potential pathogenic mechanisms underlying Fragile X Tremor ataxia syndrome: RAN translation and/or RNA gain-of-function? *Eur J Med Genet.* (2018) 61:674–9. doi: 10.1016/j.ejmg.2017.11.001
25. Ma L, Herren AW, Espinal G, Randol J, McLaughlin B, Martinez-Cerdeño V, et al. Composition of the intranuclear inclusions of Fragile X-associated Tremor/Ataxia syndrome. *Acta Neuropathol Commun.* (2019) 7:143. doi: 10.1186/s40478-019-0796-1
26. Hagerman PJ, Hagerman RJ. Fragile X-associated tremor/ataxia syndrome. *Ann N Y Acad Sci.* (2015) 1338:58–70. doi: 10.1111/nyas.12693
27. Salcedo-Arellano MJ, Dufour B, McLennan Y, Martinez-Cerdeno V, Hagerman R. Fragile X syndrome and associated disorders: clinical aspects and pathology. *Neurobiol Dis.* (2020) 136:104740. doi: 10.1016/j.nbd.2020.104740
28. Allen EG, Charen K, Hipp HS, Shubeck L, Amin A, He W, et al. Clustering of comorbid conditions among women who carry an FMR1 premutation. *Genet Med.* (2020) 22:758–66. doi: 10.1038/s41436-019-0733-5
29. Hagerman RJ, Protic D, Rajaratnam A, Salcedo-Arellano MJ, Aydin EY, Schneider A. Fragile X-associated neuropsychiatric disorders (FXAND). *Front Psychiatry.* (2018) 9:564. doi: 10.3389/fpsyt.2018.00564
30. Ross-Inta C, Omanska-Klusek A, Wong S, Barrow C, Garcia-Arocena D, Iwahashi C, et al. Evidence of mitochondrial dysfunction in fragile X-associated tremor/ataxia syndrome. *Biochem J.* (2010) 429:545–52. doi: 10.1042/BJ20091960
31. Alvarez-Mora MI, Rodriguez-Revenga L, Madrigal I, Guitart-Mampel M, Garrabou G, Milà M. Impaired mitochondrial function and dynamics in the pathogenesis of FXTAS. *Mol Neurobiol.* (2017) 54:6896–902. doi: 10.1007/s12035-016-0194-7
32. Napoli E, Song G, Wong S, Hagerman R, Giulivi C. Altered ze FMR1 premutation before the onset of the neurodegenerative disease fragile X-associated tremor/ataxia syndrome. *Cerebellum.* (2016) 15:552–64. doi: 10.1007/s12311-016-0779-8
33. Alvarez-Mora MI, Podlesniy P, Gelpi E, Hukema R, Madrigal I, Pagonabarraga J, et al. Fragile X-associated tremor/ataxia syndrome: regional decrease of mitochondrial DNA copy number relates to clinical manifestations. *Genes Brain Behav.* (2019) 18:e12565. doi: 10.1111/gbb.12565
34. Ariza J, Steward C, Rueckert F, Widdison M, Coffman R, Afjei A, et al. Dysregulated iron metabolism in the choroid plexus in fragile X-associated tremor/ataxia syndrome. *Brain Res.* (2015) 1598:88–96. doi: 10.1016/j.brainres.2014.11.058
35. Napoli E, Schneider A, Hagerman R, Song G, Wong S, Tassone F, et al. Impact of FMR1 premutation on neurobehavior and bioenergetics in young monozygotic twins. *Front Genet.* (2018) 9:338. doi: 10.3389/fgene.2018.00338
36. Napoli E, McLennan YA, Schneider A, Tassone F, Hagerman RJ, Giulivi C. Characterization of the metabolic, clinical and neuropsychological phenotype of female carriers of the premutation in the X-Linked FMR1 gene. *Front Mol Biosci.* (2020) 7:302. doi: 10.3389/fmolb.2020.578640
37. Kaplan ES, Cao Z, Hulsizer S, Tassone F, Berman RF, Hagerman PJ, et al. Early mitochondrial abnormalities in hippocampal neurons cultured from Fmr1 pre-mutation mouse model. *J Neurochem.* (2012) 123:613–21. doi: 10.1111/j.1471-4159.2012.07936.x
38. Napoli E, Ross-Inta C, Song G, Wong S, Hagerman R, Gane LW, et al. Premutation in the Fragile X Mental Retardation 1 (FMR1) gene affects maternal zn-milk and perinatal brain bioenergetics and scaffolding. *Front Neurosci.* (2016) 10:159. doi: 10.3389/fnins.2016.00159
39. Loesch DZ, Annesley SJ, Trost N, Bui MQ, Lay ST, Storey E, et al. Novel blood biomarkers are associated with white matter lesions in Fragile X- associated tremor/ataxia syndrome. *Neurodegener Dis.* (2017) 17:22–30. doi: 10.1159/000446803
40. Loesch DZ, Trost N, Bui MQ, Hammersley E, Lay ST, Annesley SJ, et al. The spectrum of neurological and white matter changes and premutation status categories of older male carriers of the FMR1 Alleles are linked to genetic (CGG and FMR1 mRNA) and cellular stress (AMPK) markers. *Front Genet.* (2018) 9:531. doi: 10.3389/fgene.2018.00531
41. Herzig S, Shaw RJ, AMPK guardian of metabolism and mitochondrial homeostasis. *Nat Rev Mol Cell Biol.* (2018) 19:121–35. doi: 10.1038/nrm.2017.95
42. Hindupur SK, González A, Hall MN. The opposing actions of target of rapamycin and AMP-activated protein kinase in cell growth control. *Cold Spring Harb Perspect Biol.* (2015) 7:a019141. doi: 10.1101/cshperspect.a019141
43. Ling NXY, Kaczmarek A, Hoque A, Davie E, Ngoei KRW, Morrison KR, et al. mTORC1 directly inhibits AMPK to promote cell proliferation under nutrient stress. *Nat Metab.* (2020) 2:41–9. doi: 10.1038/s42255-019-0157-1
44. Fahn S, Elton R, UPDRS Development Committee. "The Unified Parkinson's disease rating scale," In: Fahn S, Marsden C, Calne D, Goldstein M, editors. *Recent Developments in Parkinson's Disease*. Florham Park, NJ: Macmillan Healthcare Information (1987) p. 153-63, 293–304.
45. Trouillas P, Takayanagi T, Hallett M, Currier RD, Subramony SH, Wessel K, et al. International cooperative ataxia rating scale for pharmacological assessment of the cerebellar syndrome. the ataxia neuropharmacology committee of the world federation of neurology. *J Neurol Sci.* (1997) 145:205–11. doi: 10.1016/S0022-510X(96)00231-6
46. Fahn S, Tolosa E, Marin C. Clinical rating scale for tremor. In: Jankovic J, Tolosa E, editors. *Parkinson's Disease and Movement Disorders*. Baltimore: Williams & Wilkins; (1993). p. 271–80.
47. Mioshi E, Dawson K, Mitchell J, Arnold R, Hodges JR. The Addenbrooke's Cognitive Examination Revised (ACE-R): a brief cognitive test battery for dementia screening. *Int J Geriatr Psychiatry.* (2006) 21:1078–85. doi: 10.1002/gps.1610
48. Wechsler D. *The Wechsler Adult Intelligence Scale* (3rd ed.) Administration and Scoring Manual. Orlando: The Psychological Corporation (1997).
49. Smith A. *Symbol Digit Modalities Test*. Los Angeles, CA: Western Psychological Services. (1982).
50. Brandt J. The hopkins verbal learning test: development of a new memory test with six equivalent forms. *Clin Neuropsychol.* (1991) 5:125–42. doi: 10.1080/13854049108403297
51. Derogatis L. *SCL-90-R: Symptom checklist-90-R. Administration, scoring and procedures manual 3 edn.* Minneapolis: NCS Pearson. (1994).
52. Tassone F, Pan R, Amiri K, Taylor AK, Hagerman PJ, A. rapid polymerase chain reaction-based screening method for identification of all expanded alleles of the fragile X (FMR1) gene in newborn and high-risk populations. *J Mol Diagn.* (2008) 10:43–9. doi: 10.2353/jmoldx.2008.070073
53. Filipovic-Sadic S, Sah S, Chen L, Krosting J, Sekinger E, Zhang W, et al. A novel FMR1 PCR method for the routine detection of low abundance expanded alleles and full mutations in fragile X syndrome. *Clin Chem.* (2010) 56:399–408. doi: 10.1373/clinchem.2009.136101
54. Annesley SJ, Lay ST, De Piazza SW, Sanislav O, Hammersley E, Allan CY, et al. Immortalized Parkinson's disease lymphocytes have enhanced mitochondrial respiratory activity. *Dis Model Mech.* (2016) 9:1295–305. doi: 10.1242/dmm.025684
55. Missailidis D, Annesley SJ, Allan CY, Sanislav O, Lidbury BA, Lewis DP, et al. An isolated complex v inefficiency and dysregulated mitochondrial function in immortalized lymphocytes from ME/CFS Patients. Int J Mol Sci. (2020) 21:1074. doi: 10.3390/ijms21031074
56. Stapleton D, Mitchelhill KI, Gao G, Widmer J, Michell BJ, Teh T, et al. Mammalian AMP-activated protein kinase subfamily. *J Biol Chem.* (1996) 271:611–4. doi: 10.1074/jbc.271.2.611
57. Hall DA, Birch RC, Anheim M, Jønch AE, Pintado E, O'Keefe J, et al. Emerging topics in FXTAS. *J Neurodev Disord.* (2014) 6:31. doi: 10.1186/1866-1955-6-31
58. Napoli C, Paolisso G, Casamassimi A, Al-Omran M, Barbieri M, Sommese L, et al. Effects of nitric oxide on cell proliferation: novel insights. *J Am Coll Cardiol.* (2013) 62:89–95. doi: 10.1016/j.jacc.2013.03.070
59. Song G, Napoli E, Wong S, Hagerman R, Liu S, Tassone F, et al. Altered redox mitochondrial biology in the neurodegenerative disorder fragile x-tremor/ataxia syndrome: use of antioxidants in precision medicine. *Mol Med.* (2016) 22:548–59. doi: 10.2119/molmed.2016.00122
60. Napoli E, Song G, Schneider A, Hagerman R, Eldeeb MAAA, Azarang A, et al. Warburg effect linked to cognitive-executive deficits in FMR1 premutation. *FASEB J.* (2016) 30:3334–51. doi: 10.1096/fj.201600315R

61. Annesley SJ, Fisher PR. (2021). Lymphoblastoid cell lines as models to study mitochondrial function in neurological disorders. *Int J Mol Sci.* (2021) 22:4536. doi: 10.3390/ijms22094536
62. Hocking DR, Loesch DZ, Trost N, Bui MQ, Hammersley E, Francis D, et al. Total and regional white matter lesions are correlated with motor and cognitive impairments in carriers of the FMR1 premutation. *Front Neurol.* (2019) 10:832. doi: 10.3389/fneur.2019.00832
63. Martínez Cerdeño V, Hong T, Amina S, Lechpammer M, Ariza J, Tassone F, et al. Microglial cell activation and senescence are characteristic of the pathology FXTAS. *Mov Disord.* (2018) 33:1887–94. doi: 10.1002/mds.27553
64. Almolda B, González B, Castellano B. Are microglial cells the regulators of lymphocyte responses in the CNS? *Front Cell Neurosci.* (2015) 9:440. doi: 10.3389/fncel.2015.00440
65. Hardie DG. Keeping the home fires burning: AMP-activated protein kinase. *J R Soc Interface.* (2018) 15:138. doi: 10.1098/rsif.2017.0774
66. Garcia-Arocena D, Yang JE, Brouwer JR, Tassone F, Iwahashi C, Berry-Kravis EM, et al. Fibroblast phenotype in male carriers of FMR1 premutation alleles. *Hum Mol Genet.* (2010) 19:299–312. doi: 10.1093/hmg/ddp497
67. Arocena DG, Iwahashi CK, Won N, Beilina A, Ludwig AL, Tassone F, et al. Induction of inclusion formation and disruption of lamin A/C structure by premutation CGG-repeat RNA in human cultured neural cells. *Hum Mol Genet.* (2005) 14:3661–71. doi: 10.1093/hmg/ddi394
68. Vancura A, Nagar S, Kaur P, Bu P, Bhagwat M, Vancurova I. Reciprocal regulation of AMPK/SNF1 and protein acetylation. *Int J Mol Sci.* (2018) 19:3314. doi: 10.3390/ijms19113314
69. Todd PK, Oh SY, Krans A, Pandey UB, Di Prospero NA, Min K-T, et al. histone deacetylases suppress CGG repeat–induced neurodegeneration *via* transcriptional silencing in models of fragile X tremor ataxia syndrome. *PLoS Genet.* (2010) 6:e1001240. doi: 10.1371/journal.pgen.1001240
70. Bokko PB, Francione L, Bandala-Sanchez E, Ahmed AU, Annesley SJ, Huang X, et al. Diverse cytopathologies in mitochondrial disease are caused by AMP-activated protein kinase signaling. *Mol Biol Cell.* (2007) 18:1874–86. doi: 10.1091/mbc.e06-09-0881
71. Jeon SM, Hay N. The double-edged sword of AMPK signaling in cancer and its therapeutic implications. *Arch Pharm Res.* (2015) 38:346–57. doi: 10.1007/s12272-015-0549-z
72. Steinberg GR, Carling D. AMP-activated protein kinase: the current landscape for drug development. *Nat Rev Drug Discov.* (2019) 18:527–51. doi: 10.1038/s41573-019-0019-2
73. Rabanal-Ruiz Y, Otten EG, Korolchuk VI. mTORC1 as the main gateway to autophagy. *Essays Biochem.* (2017) 61:565–84. doi: 10.1042/EBC20170027
74. Manford AG, Rodríguez-Pérez F, Shih KY, Shi Z, Berdan CA, Choe M, et al. A cellular mechanism to detect and alleviate reductive stress. *Cell.* (2020) 183:46–61. doi: 10.1016/j.cell.2020.08.034
75. Xiao W, Loscalzo J. Metabolic responses to reductive stress. *Antioxid Redox Signal.* (2020) 32:1330–47. doi: 10.1089/ars.2019.7803
76. Fisher PR, Allan CY, Sanislav O, Atkinson A, Ngoei KRW, Kemp BE, et al. Relationships between mitochondrial function, AMPK, and TORC1 signaling in lymphoblasts with premutation alleles of the FMR1 gene. *Int J Mol Sci.* (2021) 22:10393. doi: 10.3390/ijms221910393
77. Lu C, Lin L, Tan H, Wu H, Sherman SL, Gao F, et al. Fragile X premutation RNA is sufficient to cause primary ovarian insufficiency in mice. *Hum Mol Genet.* (2012) 21:5039–47. doi: 10.1093/hmg/dds348
78. Masini D, Bonito-Oliva A, Bertho M, Fisone G. Inhibition of mTORC1 signaling reverts cognitive and affective deficits in a mouse model of Parkinson's Disease. *Front Neurol.* (2018) 9:208. doi: 10.3389/fneur.2018.00208
79. Loesch DZ, Duffy DL, Martin NG, Tassone F, Atkinson A, Storey E. 'Essential tremor' phenotype in FMR1 premutation/gray zone sibling series: exploring possible genetic modifiers. *Twin Res Hum Genet.* (2021) 24:95–102. doi: 10.1017/thg.2021.10
80. Roberts JE, Bailey DB, Jr., Mankowski J, Ford A, Sideris J, Weisenfeld LA, et al. Mood and anxiety disorders in females with the FMR1 premutation. *Am J Med Genet B Neuropsychiatr Genet.* (2009) 150b:130–9. doi: 10.1002/ajmg.b.30786
81. Bourgeois JA, Seritan AL, Casillas EM, Hessl D, Schneider A, Yang Y, et al. Lifetime prevalence of mood and anxiety disorders in fragile X premutation carriers. *J Clin Psychiatry.* (2011) 72:175–82. doi: 10.4088/JCP.09m05407blu
82. Bourgeois JA, Coffey SM, Rivera SM, Hessl D, Gane LW, Tassone F, et al. A review of fragile X premutation disorders: expanding the psychiatric perspective. *J Clin Psychiatry.* (2009) 70:852–62. doi: 10.4088/JCP.08r04476
83. Loesch DZ, Bui MQ, Hammersley E, Schneider A, Storey E, Stimpson P, et al. Psychological status in female carriers of premutation FMR1 allele showing a complex relationship with the size of CGG expansion. *Clin Genet.* (2015) 87:173–8. doi: 10.1111/cge.12347

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Maternal Mental Health and Parenting Stress and Their Relationships to Characteristics of the Child With Fragile X Syndrome

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Although previous research supports the notion that characteristics of both the child and the mother impact maternal well-being and parenting stress in mothers of children with FXS, more work is needed in which self-report measures are supplemented by physiological measures of stress and well-being. The inclusion of physiological measures, such as heart rate variability (HRV), may provide a window into the biological origins and consequences of maternal perceptions of their experiences, including the challenges of raising a child with developmental challenges. The proposed project, therefore, involved the collection of multimodal assessment data from mothers and their school-aged children with FXS. Further, given the importance of understanding how mothers of youth with FXS are faring in their everyday environment, the present study collected all data using telehealth procedures and wearable technology. Participants were 20 biological mothers and their children with FXS between the ages of 6 and 11 years. We measured maternal mental health and parenting stress through self-report as well as through HRV as a more objective measure of psychological well-being. We also examined the associations between these variables and child characteristics such as externalizing and internalizing behaviors as well as autism symptomatology. We found significant support for an elevated rate of depressive symptoms in the sample of mothers (35%) and some potential indicators for heightened rates of anxiety (15%) when compared to normed samples and rates in the general population. We also found that the mothers presented with an atypical HRV profile akin to those experiencing depression or chronic stress, although limitations of the present measure suggest the need for additional confirmatory research. Further, we found that child externalizing behaviors were the primary correlates of maternal well-being. These findings contribute to the growing body of literature regarding the unique challenges faced by these mother-child dyads and supports the importance of increasing the availability of services available to these mothers, not only for meeting the needs of their children's development and behavior, but in supporting their own well-being as well.

Keywords: ***FMR1*** **carriers, fragile X syndrome, mental health, parenting stress, telehealth**

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INTRODUCTION

Considerable research has shown the importance of parenting in supporting typically developing (TD) children's development across multiple domains, including academic achievement and social emotional development (1, 2). Parenting also contributes to the development of children with neurodevelopmental disabilities, such as those with autism spectrum disorder [ASD; (3)], Down syndrome [DS; (4)], or fragile X syndrome [FXS; (5–7)]. However, parents of children with neurodevelopmental disabilities face challenges to parenting, such as increased effort to help their child achieve daily tasks as well as pessimism regarding their child's future compared to parents of TD children (8, 9). Moreover, these parents are often engaged in high levels of advocacy on behalf of their children from birth [or receipt of diagnosis; (10)] and persists through adulthood as they advocate for job placements and more (11). These parents often shoulder additional financial demands surrounding the cost of specialized therapies and health care for their children (12). Parents of children with neurodevelopmental disabilities may also be required to deal with child challenging behaviors, such as aggression and hyperarousal (13, 14), limitations in child adaptive functioning (15–17), as well as social (18, 19) and academic challenges (20, 21), all of which can further contribute to higher caregiver burden and stress. At the same time, however, there are between-syndrome differences in how parents fare; for example, parents of children with FXS often experiencing higher rates of parenting stress and greater impacts to their mental health and well-being relative to parents whose children have Down syndrome [e.g., (8, 22)]. In the case of biological mothers of children with FXS, they are carriers of the genetic alteration that is the source of their children's FXS and a subset may themselves be at an increased risk for anxiety and depression or more subtle symptoms of emotionality such as negative affect in part because of carrying the alteration although genetic factors play a moderating role as well (23–26). Thus, biological mothers of children with FXS are at risk for poorer mental health and stress, which could affect their parenting, by virtue of both their own genetic vulnerabilities and their children's characteristics. These mother-child dyads could benefit from increased support and services to help achieve optimal outcomes for the entire family system. The study reported here focused on understanding the sources of stress and poor psychological well-being in biological mothers of children with FXS to inform the development and delivery of support and services.

Genetics of Fragile X Syndrome

FXS is the leading inherited cause of intellectual disability and the leading single-gene cause of autism spectrum disorder [ASD; (27, 28)]. FXS is one of several conditions resulting from repetitions of the trinucleotide sequence, CGG, of the fragile X mental retardation 1 (*FMR1*) gene (29). The length of the CGG repeat expansion can ultimately impact neuronal functioning by impacting production of the Fragile X Mental Retardation Protein (FMRP). Expansions between 55 and 200 are considered *premutations*, which occur in 1 in 151 females and is somewhat less common in males (30). Individuals with expansions of 200 or more have the *full mutation* and are most likely to display the behavioral and physical features of FXS (31). Males with the full mutation are more affected than females, on average, because females benefit from the protective effects of a second unaffected X chromosome (32).

Although premutation carriers do not typically have an intellectual disability or display the challenging behaviors associated with FXS, they do have their own unique phenotypic features. Female carriers of the *FMR1* premutation, for example, are more likely to experience mental health related issues such as anxiety and depression (33), as well as physical health complications such as early onset of menopause, infertility, and irregular menstrual cycles reflecting fragile X-associated primary ovarian insufficiency [FXPOI; (34, 35)]. Thus, these women present with a complex physical and emotional phenotypic profile that warrants treatment and support, especially when considering the added impacts of parenting a child with special needs.

Psychological Well-Being and Parenting Stress in Biological Mothers of Children With Fragile X Syndrome

Individual mental health affects the quality of interactions within a family (36). Biological mothers of individuals with FXS themselves have either the *FMR1* full mutation or, more commonly, the *FMR1* premutation. Thus, a subset of these mothers are at increased risk for mental health concerns, such as depression (37) and anxiety (38, 39), which can affect their parenting. In addition, biological mothers of youth with FXS are more pessimistic about the youth's future, perceive less reciprocated closeness in the parent-offspring relationship, and display more symptoms of depression (8). Mothers of children with FXS have also self-reported lower quality of life (40). Importantly, there is variability among mothers with the *FMR1* premutation in symptom presentation, which may be attributed in part to individual differences in genetic susceptibility and differences in their responses to the stress of raising a child with a disability (25, 41). Moreover, there are biological and social determinants that might explain at least some of the variability in symptom expression including cognitive abilities and life stressors (24, 25, 42). Thus, understanding the source(s) of lowered maternal well-being beyond genetic susceptibility is important for determining optimal paths toward treatment (40).

Children with FXS often display challenging behaviors, such as those consistent with a diagnosis of ASD (43–45), restricted and repetitive behaviors (46, 47), self-injurious behaviors (48), as well as increased rates of anxiety (49, 50). These co-occurring problems impact levels of parenting stress and parental well-being over time (5, 8, 13, 22, 26). Further, high levels of parenting stress may exist throughout the course of the child's development, starting at the time of diagnosis and continuing well into adolescence and adulthood (51). This heightened and prolonged experience of stress can have lasting impacts on the overall health of parents (52, 53). *FMR1* premutation carriers are at an increased risk for developing additional physical health issues, including thyroid disease, hypertension, seizures, peripheral neuropathy,

and fibromyalgia (54), which may also contribute to parental stress. Thus, it is important to understand the extent, sources, and consequences of stress experienced by parents to determine ways to better promote health and well-being.

Measuring Parenting Stress in Mothers of Youth With FXS

Although the vast majority of studies characterizing parenting stress in studies of FXS has involved self-report measures (13, 55), self-report is non-objective and more likely to lead to spurious relationships compared to more objective measures of stress, such as physiological indicators (56). Therefore, in recent years there has been increasing research exploring whether more objective indices of stress, such as physiological markers like cortisol (57, 58), electrodermal activity [EDA; (59)], and heart rate measurements (60, 61) can help us better understand the health and well-being of parents of youth with neurodevelopmental disabilities (62, 63). Measures of the functioning of the autonomic nervous system (ANS) have often been targeted due to its impact on overall health and its role in support of emotional regulation (64) and stress management (65). ANS health and functioning is often indexed by the measurement of heart rate variability (HRV). As opposed to heart rate, which is the continuous beat of the heart, HRV is the time in between heart beats. HRV can be considered under a baseline, or non-stressful at-rest, condition, as well as under a stressful situation for an index of stress reactivity. High HRV at rest reflects overall positive health and optimal emotional regulation (66, 67), and has been associated with increased responsiveness and adaptation to the environment (68). In otherwise healthy individuals, a low HRV at rest could be an indicator of depression (66, 69, 70) or chronic stress (71, 72). Further, the default physiological stress response is characterized by an increase in heart rate, and thus a decrease in HRV, after being exposed to a stressor before ultimately returning to baseline levels (73). Importantly, maladaptive stress responses have been linked to depression (69) and anxiety (74).

There is limited evidence on how HRV and parenting stress are related in parents of individuals with neurodevelopmental disabilities. In a recent study, however, Factor et al. (60) found preliminary support for an atypical stress response as measured by HRV during interactions between mothers and their children with ASD. Specifically, Factor et al. found a positive association between child ASD symptoms and mothers change in HRV, such that a higher rate of symptoms of ASD was related to a larger increase in HRV from baseline. In terms of *FMR1* premutation carriers, a study by Klusek et al. (75) quantified respiratory sinus arrhythmia, an estimate of vagal tone and a metric comparable to HRV, and found support for ANS dysregulation through the observation of reduced vagal tone when compared to study controls. Identifying and treating atypical ANS functioning is especially critical given its relationship to long term health outcomes and therefore should be explored further in this already vulnerable population of *FMR1* carriers. For instance, more research is needed to understand how ANS dysregulation in premutation carriers might be impacted due to parenting stress in particular as well as across other measures of ANS processes which might yield different insights into this complex system. It is also of importance to understand how these mechanisms work in relation to more real-world stressors and interactions such as those between the mother and their child.

Current Study

Although previous research supports the notion that characteristics of both the child and the mother impact maternal psychological well-being and parenting stress in mothers of children with FXS, more work is needed in which self-report measures are supplemented by physiological measures of stress and well-being. The inclusion of physiological measures, such as the HRV measures used in this study, may provide a window into the biological origins and consequences of maternal perceptions of their experiences, including the challenges of raising a child with developmental delays. The present study used multimodal assessment data, including physiological measures, from mothers and their school-aged children with FXS to further understand maternal mental health, stress, and well-being as they relate to child characteristics. We focused on school-aged children given evidence that parenting stress is highest when children with disabilities are between the ages of roughly 6–11 or 12 years (76, 77). Further, given the importance of understanding how mothers of youth with FXS are faring in their everyday environment, we collected data through online questionnaires, wearable physiological wristbands, interviews with the mother as well as direct assessment of maternal cognitive abilities, and real-time observations of mother-child interactions in the home by means of distance teleconferencing. This form of data collection allowed us to gain more naturalistic and ultimately more generalizable data as well as eased the burden on the families with regards to needing to travel for participation. Two primary hypotheses were addressed:

(1) Given their genetic status as carriers of the *FMR1* premutation or full mutation (26), biological mothers of children with FXS, on average, were expected to display elevated rates of mental health symptoms, especially depression and anxiety (39), and parenting stress (41) relative to the general population. We tested this hypothesis using not only self-report measures of well-being but also objective physiological measurement (HRV), making it possible to determine the correspondence between maternal perceptions and physiological indices. We also examined variability in such symptoms among the mothers.
(2) Increased maternal mental health symptoms, higher maternal-reported parenting stress (8), and atypical ANS regulation, as measured by HRV (60, 75), were expected to be associated with increased child challenging behavior and symptoms of ASD.

MATERIALS AND METHODS

Participants

Twenty-two mother-child dyads were recruited to participate in a larger study profiling the characteristics of mothers and children

with FXS and how these characteristics impact the mother-child interaction. Of the 22 dyads enrolled, 2 discontinued prior to starting data collection and one discontinued after completing only the study questionnaires detailed below. All mothers provided electronic informed consent on behalf of themselves as participants and for their participating child prior to beginning data collection. Families were recruited from around the United States and from Canada through community listservs, university research volunteer registries, and existing lab databases or previous participants. Child participants had a confirmed diagnosis of the full mutation FXS, documented through diagnostic reports shared by parents, and were between 6 and 11 years of age. Both male and female children were recruited to participate (16 males and 4 females) along with their biological mothers. The sample was racially diverse with child participants distributed as follows: 55% White, Non-Hispanic/Latinx, 5% American Indian/Alaskan Native, 5% Black or African American, 15% Hispanic/Latinx, and 20% were multicultural (with 3 of the 4 multicultural families identifying as both Black/African American and White and 1 identifying as American Indian/Alaskan Native and White). Additional eligibility criteria for the child participants were that the child lived at home with the biological mother and that English was the primary language used in the home.

Biological mothers were recruited due to their genetic susceptibility to mental health challenges and increased rates of parenting stress. The participating mothers self-reported on their *FMR1* carrier status, with two mothers having the full mutation, 16 had the premutation, and two never received testing and, therefore, did not know their carrier status. Mothers ranged in age from 28 to 47 years and in IQ from 81 to 131 on the General Ability Measure for Adults [GAMA; (78)]. Participating mothers were 60% White, Non-Hispanic/Latinx, 5% Black or African American, 15% were Hispanic/Latinx, and 20% were multicultural (with 50% indicating both American Indian/Alaskan Native and White and the other 50% indicating both Black/African American and White). Household incomes for the participating families ranged from between 30,001 and 35,000 annually to more than 300,000 annually. For a complete summary of participant characteristics and household information (see **Table 1**). Overall, the current study sample reflects a largely educated and well-resourced sample of mothers, which is a consistent limitation reported in other studies of this kind in this population [e.g., (5)]; however, the present sample is more culturally diverse than is typical.

TABLE 1 | Study participant and household demographics.

	N	%
Child participants (6–11 years old; *M* = 8.80, *SD* = 1.77)		
Female	4	20
Male	16	80
American Indian/Alaskan Native	1	5
Black or African American	1	5
Hispanic/Latinx	3	15
White, Not Hispanic/Latinx	11	55
More than one race/ethnicity*	4	20
Mother participants (28–47 years old; *M* = 40.35, *SD* = 5.27)		
American Indian/Alaskan Native	0	0
Black or African American	1	5
Hispanic/Latinx	3	15
White, Not Hispanic/Latinx	12	60
More than one race/ethnicity*	4	20
Household information		
Household income		
< $50,000	1	5
$50,000–$150,000	9	45
> $150,000	9	45
Preferred not to answer	1	5
Caregiver status		
Primary caregiver (mother)	20	100
Two parent/caregiver household	16	80
One parent/caregiver household	4	20

**75% of the child participants that selected more than once race families endorsed both Black/African American and White, with the remaining 25% indicating American Indian/Alaskan Native and White. 50% of mothers reporting more than one race selected Black/African American and White and the other half selected American Indian/Alaskan Native and White.*

Procedures

Families were asked to use their own technology for video teleconferencing (i.e., personal tablets or computer). However, in cases in which a family did not have access to technology of their own, we provided a device equipped with video teleconferencing software (e.g., Skype for Business™) and the other applications required to complete the assessments from a distance. Most of the mothers elected to use their own technology, with only two mothers needing technology to be provided. All mothers were provided an Empatica E4 physio-wristband to assess physiological data of the mother in real time. In addition to the physio-wristbands, a wordless picture book and standardized assessment materials were provided. Upon receiving the materials, study staff conducted a technology training session to orient the mother to the technology to be used and to set-up the home environment for optimal data collection. Data were collected by the first author (LB) through a combination of mother-completed online questionnaires, mother interviews, remotely conducted direct assessment of maternal cognitive ability, physiological data, and direct observations of mother-child interactions. Data collection occurred across several distance sessions. All technology applications were HIPAA compliant and, when provided, all computers were encrypted to the specification of the UC Davis Health System.

Child Measure

In order to provide a comprehensive view of the child factors that were hypothesized to impact maternal well-being and parenting stress, the below measures were collected (**Table 2**). Measures

TABLE 2 | Means and standard deviations for child and maternal measures.

	M	*SD*
Child measures		
Vineland-3		
Adaptive behavior composite score	67.11	18.66
Childhood autism rating scale (raw score)		
Autism symptom severity	28.74	6.17
Child behavior checklist for ages 6–18 (T-scores)		
Internalizing behaviors	57.10	8.012
Externalizing behaviors	53.00	9.42
Total behaviors	59.85	9.31
Maternal measures		
General ability measure for adults		
GAMA IQ score	108.26	12.06
Symptom checklist 90-revised (T-scores)		
Anxiety	50.55	10.70
Depression	57.15	9.47
Global severity index	54.80	12.06
Parenting stress index, fourth edition (T-scores)		
Child domain	62.55	11.88
Parent domain	53.20	9.26
Total parenting stress	58.20	10.68
Heart rate variability (millisecond)		
Baseline HRV	50.09	16.04
Interaction HRV	67.37	22.34
Change in HRV	17.17	15.15

of child language ability were also derived from the mother-child narratives; however, those measures are not included in this report.

Vineland-3

The Vineland-3 (79) was used to assess child adaptive functioning. The Vineland-3 provides scores for specific adaptive behavior domains (i.e., Communication, Daily Living Skills, and Socialization, as well as an overall adaptive behavior composite). The Vineland-3 meets American Association on Intellectual and Developmental Disabilities and DSM-5 requirements for identifying impairments in adaptive behavior as one key component in identifying an intellectual disability. The Vineland-3 was administered as an interview with the mother *via* distance video teleconferencing. For the purposes of the current study, we used the adaptive behavior composite standard score as a proxy for child developmental level, with scores in the present sample ranging from 40 to 127 ($M = 67.11$, $SD = 18.66$). Higher scores reflect more advanced developmental levels. One mother-child dyad discontinued with only partial data collection; therefore, the Vineland-3 was only collected on 19 of the youth participants.

Childhood Autism Rating Scale, Second Edition (CARS-2)

Child symptoms of ASD were also assessed using the CARS-2 (80), which is a measure used to identify children with autism and determine symptom severity through quantifiable ratings based on direct observation. The CARS-2 format lends itself readily to use *via* telehealth. The CARS-2 was coded by trained research staff from the recorded mother-child interaction in which the mother and child told a wordless picture book together. Child total scores on the CARS-2 were used as an indicator of symptom severity (with scores between 15 and 29 reflecting minimal-to-no symptoms of ASD, scores between 30 and 36.5 reflecting mild-to-moderate symptoms of ASD, and scores of 37 and higher reflecting severe symptoms of ASD). Scores in the present sample of youth ranged between 16 and 42 ($M = 28.74$, $SD = 6.17$). One mother-child dyad discontinued with only partial data collection; therefore, the CARS-2 was only scored for 19 participants.

Child Behavior Checklist for Ages 6–18 (CBCL:6-18)

The CBCL (81) is a parent-report measure of child challenging behaviors and was used to assess child challenging behaviors. The measure creates a composite T-score of both externalizing and internalizing behaviors as well as an overall total challenging behavior T-score. The CBCL was collected on all 20 of the youth participants with scores ranging between 39 and 75 for internalizing ($M = 57.10$, $SD = 8.01$), 34 and 69 for externalizing ($M = 53$, $SD = 9.42$) and between 41 and 74 for total behaviors ($M = 59.85$, $SD = 9.31$).

Maternal Measures

In order to provide a comprehensive view of maternal factors we collected information regarding mental health status as well as stress (self-reported and physiological, see **Table 2**), in addition to the more descriptive characteristics such as *FMR1* carrier status and cognitive ability described previously:

General Ability Measure for Adults (GAMA; Naglieri and Bardos, 1997)

The GAMA (78) is a 66-item, self-administered timed test (e.g., 25 min) that assesses general cognitive ability of individuals 18 years and older and has been redesigned for implementation *via* telepractice. For this study the mother and research staff (LB) connected *via* video teleconferencing with LB proctoring the administration in real-time. The GAMA was collected on 19 mothers.

Symptom Checklist 90-Revised (SCL-90-R)

The SCL-90-R (82) is a 90-item informant report of current psychological problems and symptoms of psychopathology. For each subscale as well as the Global Severity Index (GSI), scores are reported based on a T score distribution with a mean of 50 and standard deviation of 10. T-scores that are $\geq$63 are considered to be in the clinically significant range (82). In light of the expected phenotype of premutation carriers, we used scores only from the anxiety and depression subscales. The SCL-90-R was adapted to an online questionnaire format and collected for all 20 of the participating mothers.

Parenting Stress Index, Fourth Edition (PSI-4)

The PSI-4 (83) is a 120-item informant report questionnaire to be completed by the mother. It contains three major domains of stress: child characteristics, parent characteristics, and situational/demographic life stress. Percentile scores from the PSI-4 are commonly used to interpret clinical status of the

parent informant; however, T-scores are also provided based on a normal distribution with a mean of 50 and standard deviation of 10 and are used in subsequent analyses. The PSI-4 was adapted to an online questionnaire format and was collected for all 20 of the participating mothers.

Physiological Markers of Stress

Empatica E4 wristbands were used to collect physiological data as a marker of parenting stress in real time. The E4 wristband is a wearable research device designed to collect reliable metrics of stress and health physiology through heart rate measurements and electrodermal activity and is comparable to more clinical based measures including ECG monitors (84, 85). The E4 wristband is equipped with PPG sensors that measure the Blood Volume Pulse (BVP), from which heart rate variability can be derived. The PPG data allows derivation of inter-beat interval (IBI) and thus, HRV. The Empatica E4's algorithm automatically detects abnormal or anomalous heartbeats, often created by motion artifacts, and removes them prior to creating the IBI file, thereby leaving only accurate heartbeats. IBI data files were further inspected visually for any remaining anomalies in the data caused by motion artifacts. From the extracted IBI data, we computed the standard deviation of the IBI of normal sinus beats (SDNN) as our measure of HRV (86). Mothers wore the physio-wristband for 5-min during a baseline, or non-stress inducing, activity (i.e., watching a video of waves crashing on the computer), as well as during the shared telling of a wordless picture book with their child. One mother did not complete either the mother-child interactions or collect physiological data and thus is considered missing for all of the physiological data analyses. Further, one mother encountered technology issues with the wristband and was thus missing data for both contexts, and two other mothers inadvertently turned the watch off after the baseline context and resulting in missing data for the interaction context. Thus, we had useable baseline physiological data for 18 mothers and complete data (i.e., baseline and dyadic interaction with the child) for 16 mothers.

Data Analysis Plan

To address our first hypothesis, we provided descriptive summaries of symptom severity and stress profiles for the present sample of mothers and computed a series of one-sample *t*-tests using mean T-scores for the study participants against the normative samples for the measures of interest. To address our second hypothesis, we conducted a series of Pearson correlations to examine potential associations between the mother and child variables of interest. Multiple linear regressions were also computed to assess the combined impacts of child characteristics on factors of maternal well-being. Shapiro-Wilk tests of normality were conducted for the primary variables of interest. No tests yielded significant results and thus, the assumptions of normality were met and parametric tests were used in all analyses. To address the potential for Type 1 error due to multiple statistical tests, we conducted Benjamini-Hochberg False Discovery Rate correction. We present both the uncorrected significance levels and flagged significant findings that remained after correction. Analyses were conducted using SPSS statistics software.

RESULTS

Profile of Mothers Mental Health and Parenting Stress

On average, the mothers in the present sample achieved scores above the means for the general population across a variety of indices of mental health and parenting stress. On the SCL-90-R, the mothers had T-scores between 34 and 71 ($M = 57.15$, $SD = 9.47$) on the depression subscale, scores between 37 and 68 ($M = 50.55$, $SD = 10.70$) on the anxiety subscale, and scores between 37 and 68 ($M = 54.80$, $SD = 10.55$) on the Global Severity Index (GSI). Further, 35% of the mothers reported depressive symptoms consistent with a "case" (i.e., a T-score $\geq$63 reflecting symptom expression consistent with a clinical diagnosis), which is a notably higher percentage than the national average, which ranges between 10.1% in women between the ages of 20–39 years to 11.5% in women between the ages of 40–59 years (87). The mothers in the present sample also included a higher rate of anxiety "cases," with a rate of 15% compared to 3.4% for generalized anxiety disorder in the general population of women (88). We further explored how this sample of mothers compared to the normative samples used to develop the measures using *t*-tests and a comparison value of 50 as the hypothesized mean based on a normal distribution. The present sample of mothers reported significantly higher levels of depression [$t_{(19)} = 3.38$, $p < 0.01$], but not significantly higher levels of anxiety. The GSI for the mothers was marginally higher than that of the norming sample [$t_{(19)} = 2.04$, $p = 0.056$; **Figure 1**]. Moreover, there was some comorbidity of symptom expression with 15% of the mothers reporting symptoms consistent with "caseness" for both depression and anxiety. Lastly, in an exploratory analysis examining how social determinants might relate to the various features of maternal well-being, we found a significant and negative correlation between maternal IQ and depression scores [$r_{(19)} = -0.471$, $p < 0.05$].

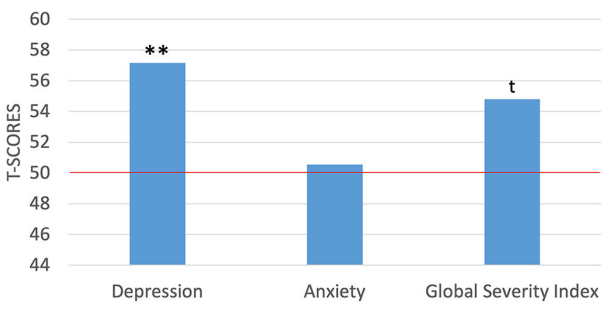

FIGURE 1 | One-sample *t*-tests between maternal self-report of mental health symptoms against the normed sample of the SCL-90-R. $^{**}p < 0.01$; *t* p = marginally significant. Red horizontal line indicates the normed sample mean of 50.

With regards to parenting stress, the mothers in the current study achieved T-scores between 38 and 91 ($M = 62.55$, $SD = 11.88$) on the child domain, between 35 and 75 ($M = 53.20$, $SD = 9.26$) on the parent domain, and scores between 36 and 86 ($M = 58.20$, $SD = 10.68$) for total parenting stress. To compare the study sample of mothers against the normative sample, we again used *t*-tests for the PSI-4 scores following the same procedures outlined above. Results from the PSI-4 indicated that the mothers in the current study reported significantly higher levels of stress in the child domain [$t_{(19)} = 4.72$, $p < 0.001$] and in total stress [$t_{(19)} = 3.43$, $p < 0.01$], but not in the parent domain than did the norming sample (**Figure 2**). Further, because percentiles are suggested for clinical interpretation on the PSI-4, we examined the distribution of percentile scores in the present sample of mothers with scores below the 85th percentile being within the normal range, scores between the 85th and 89th percentile within the high range and scores in the 90th percentile and higher being considered clinically significant. Percentile scores ranged from 9 to 99% ($M = 79.05\%$, $SD = 23.26$) for the child domain, 3 to 99% ($M = 61.95\%$, $SD = 26.43$) on the parent domain, and between 3 and 99% ($M = 72.20\%$, $SD = 23.79$) for total stress. Notably, the mothers scored within the clinically significant range at a higher than expected rate of 10% for the normative sample in two instances: 30% of the mothers scored within the clinically significant range for the child domain and 20% scored within the clinically significant range for total stress level. For the remaining PSI-4 domains, maternal scores for were more consistent with the expected distributions from the normative sample, with 15% of the mothers scoring within the high range on the child domain, 10% of the mothers scoring within the clinically significant range for the parent domain (all others were within the normal range), and 15% scoring within the high range for total stress.

The final metric of stress was HRV collected during the baseline activity (sitting while watching a video of waves crashing) and during an interaction with their child (sitting while telling a wordless picture book story with their child). There was a significant difference between the two conditions ($t = -4.241$, $p < 0.01$) with mothers in the current study having an HRV between 17.65 and 72.03 ms ($M = 50.09$ ms, $SD = 16.04$) during the baseline activity and an HRV between 30.31 and 109.94 ms ($M = 67.36$ ms, $SD = 22.34$) during the wordless picture book interaction with their child. Change in HRV between the two contexts ranged from a decrease of 15.16 ms to an increase of 48.87 ms, with a mean increase in HRV between the two contexts of 17.17 ms ($SD = 15.15$). On average, the profile of stress reactivity in the mothers in the present study contrasts with that expected for a typical and healthy stress response (73) and more comparable to individuals experiencing depression (89) or chronic stress (90).

Further, Shaffer and Ginsberg (86) suggest that individuals who have an HRV below 50 ms during a resting condition are unhealthier, those with HRV between 50 and 100 ms have compromised health, and those with HRV over 100 ms are healthy. Based on these guidelines, 44% of the mothers had a resting HRV in the unhealthy range (i.e., below 50 ms during the baseline activity), and the remaining 56% had a resting HRV in the compromised health range. In contrast, another study examining the average HRV in the general healthy population suggests an approximate HRV of 50 ms with a standard deviation of 16 (91), which is quite similar to the findings for the present sample of mothers as well.

Maternal Well-Being as It Relates to Child Characteristics

A series of two-tailed, Pearson correlations were computed to assess relationships between measures of maternal well-being and child characteristics. For self-report measures of maternal mental health and parenting stress, we found that child externalizing behaviors, as measured by the CBCL, were positively correlated with all the maternal variables of interest (see **Table 3**; **Figure 3**), and this finding remained significant after FDR correction. Further, we found that child adaptive behavior, as measured by the Vineland-3, was also significantly and negatively correlated with the child domain score on the PSI-4; however, this correlation was no longer significant after the FDR correction.

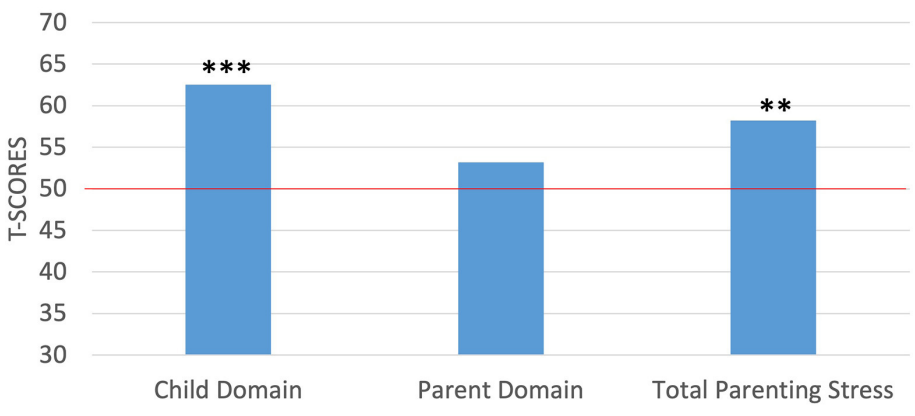

FIGURE 2 | One-sample *t*-tests between maternal self-report of parenting stress against the normed sample of the PSI-4. ***$p < 0.001$; **$p < 0.01$. Red horizontal line indicates the normed sample mean of 50.

TABLE 3 | Correlations between maternal measures of mental health and stress and child characteristics.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Parenting stress index-4															
1. Child domain	-														
2. Parent domain	**0.771****	**-**													
3. Total stress	**0.951****	**0.928****	-												
Symptom checklist 90-revised															
4. Depression	**0.608****	**0.754****	**0.718****	-											
5. Anxiety	0.534*	**0.661****	**0.626****	**0.799****	-										
6. Global severity index	**0.574****	**0.772****	**0.701****	**0.941****	**0.884****	-									
Heart rate variability															
7. Baseline HRV	−0.067	−0.020	−0.035	0.006	−0.225	−0.070	-								
8. Wordless Picture Book Interaction HRV	−0.092	0.122	0.006	−0.021	−0.244	−0.126	**0.735****	-							
9. Change in HRV	−0.059	0.209	0.062	−0.081	−0.240	−0.183	−0.005	**0.674****	-						
Child characteristics															
10. Age	0.221	0.115	0.212	0.040	0.154	0.091	0.235	−0.159	−0.411	-					
11. Adaptive functioning	−0.554*	−0.122	−0.371	0.053	−0.054	−0.031	0.220	0.254	0.217	−0.085	-				
12. Internalizing behaviors	0.082	0.054	0.054	0.199	0.053	0.244	−0.194	−0.239	−0.198	0.080	−0.094	-			
13. Externalizing behaviors	**0.780****	**0.611****	**0.733****	**0.615****	0.491*	**0.629****	0.058	−0.125	−0.294	0.253	−0.415	0.460	-		
14. Total behaviors	**0.620****	0.411	0.538*	0.503*	0.359	0.515*	−0.073	−0.189	−0.278	0.133	−0.417	**0.681****	**0.921****	-	
15. ASD symptomatology	0.388	0.014	0.229	−0.207	−0.046	−0.135	0.055	−0.240	−0.231	0.120	**−0.629****	0.050	0.224	0.222	-

***Correlation is significant at the 0.01 level (2-tailed). *Correlation is significant at the 0.05 level (2-tailed).*

The bolded correlations remained significant after accounting for multiple comparisons using the Benjamin-Hochberg procedure to detect the false discovery rate.

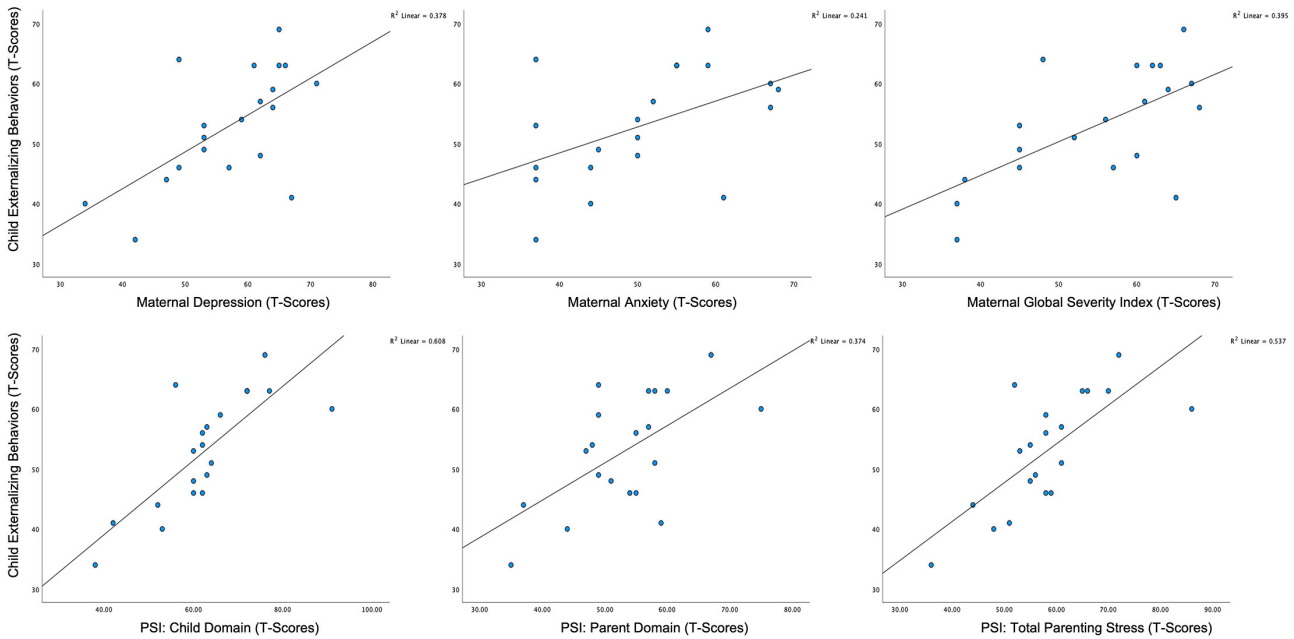

FIGURE 3 | Scatterplots of associations between child externalizing behavior and maternal mental health status and parenting stress.

To assess the combined effects of child externalizing behaviors and child adaptive functioning on the child domain scores on the PSI-4, we computed a multiple linear regression. The overall model was significant [$F_{(2,17)} = 14.239$, $p < 0.001$, $R^2 = 0.648$], with child externalizing behaviors emerging as the only unique predictor ($t = 3.89$, $p < 0.01$). There were no significant associations between child characteristics and HRV.

DISCUSSION

The present study was designed to build on previous research by exploring the phenotypic characteristics of biological mothers of children with FXS and determine how child characteristics might contribute to aspects of maternal well-being. All data were collected in the family home through various forms of technology, from video teleconferencing to digital wearables, through virtual interactions. Although preliminary because of the relatively small sample size, the findings from the current study are consistent with and extend previous findings regarding the compromised psychological well-being of at least a subset of biological mothers of children with FXS [e.g., (23, 39)], thereby suggesting that remote data collection yields similar findings as does questionnaire-based and in-person studies. This study also provided novel findings as regards to physiological measurement of stress suggesting through wearable technology to measure heart rate, demonstrating the feasibility of distance data collection though wearable technology. More generally, the findings indicate elevated rates of depression and parenting stress, as measured through both self-report and a physiological measure (HRV), in biological mothers of children with FXS as well as a relationship between child externalizing behavior and different facets of maternal psychological well-being. These findings highlight the need to address the mental health and stress facing mothers as well-target child challenging behaviors to help achieve optimal family functioning.

Profiles of Maternal Well-Being

Consistent with previous findings in the literature, the mothers in the present study had elevated rates of depression compared to the general population (33, 37). In contrast to previous studies showing higher rates of anxiety in *FMR1* carriers (38, 39), the mothers in the present study were not significantly higher in anxiety when compared to the normative sample; however, the difference relative to the general population just failed to reach statistical significance and could be attributed to a lack of power given our sample size. Thus, the risk for anxiety disorders in these women should not be ignored. Given the importance of mental health to overall physical health and well-being and to the quality of parenting, support should be made available routinely to these mothers in order to help them deal with, or even prevent, these mental health challenges. Although in the larger literature, symptoms of depression and anxiety have been shown to positively respond to pharmaceutical treatments and interventions such as seeing a counselor/therapist in the general population, the efficacy of these treatments has not been fully explored in this specific population. It is also possible that increased support (92), counseling (93), and respite services (94) provided to parents, and particularly at critical points in the development of the individual with FXS (e.g., the transition to school, the onset of puberty), could even prevent or forestall the mental health challenges experienced by these mothers. Future research should consider how we can capture the current use of

such services as well as their efficacy given the unique phenotypic characteristics and experiences of biological mothers of children with FXS.

In addition to individual mental health and well-being, because of the nature of their maternal caregiving role for children with special needs, we also explored their profiles of parenting stress. The mothers in the present study had significantly higher feelings of parenting stress, especially with regards to child-associated stressors, when compared to a normative sample of parents. This finding is also consistent with the literature in that mothers of children with neurodevelopmental disabilities such as FXS are at elevated risk of experiencing parenting stress when compared to those of more typically developing children (22, 95). Given the fact that the caregiver role for parents extends well-beyond what is typical given the high level of dependence individuals with FXS require, even into adulthood (96), these mothers are also at a risk for experiencing chronic stress that ultimately could negatively impact their overall physical as well as mental health (97). Importantly, there is evidence to suggest the importance of increasing self-care practices in such chronically stressed caregivers in supporting stress management (98, 99). Indeed, a recent study has documented the benefits of a mindfulness training intervention for *FMR1* premutation carriers (100). This potential area for intervention is particularly important given recent findings by Wheeler et al. (101), suggesting that mindfulness played a protective roll across multiple metrics of maternal well-being, including anxiety, depression, stress, and overall health. Perhaps mindfulness could be thought of as not only a way to provide coping strategies for parents experiencing elevated levels of stress and poor well-being, but also a preventive strategy to avoid poor psychological well-being in these women. Moreover, continued implementation of behavioral interventions that target child characteristics such as those found to relate to maternal well-being and parenting stress in the present sample (e.g., externalizing behaviors like irritability, aggression, etc.) are paramount to the meeting the needs of the whole family especially when parents are included in their child's treatment plan [e.g., through parent mediated interventions; (102, 103)].

In addition to self-report measures of parenting stress and well-being, a novel aspect of the present study, we also explored the role HRV plays in understanding maternal stress and overall health and well-being in this population of mothers. We considered maternal HRV across two contexts, first during a seated resting state/non-stressful activity and then during a seated interaction with their child (e.g., telling a story together) that followed immediately. From a review of the larger literature, it is thought that a higher resting HRV is indicative of physiological resilience to stressors and that further, in healthy individuals, when they are faced with a stressor, a typical profile is for HRV to decrease in response (72), although there is considerable individual variability and some inconsistency across studies in this regard (69). In the present study, mothers had relatively low HRV during the resting condition which then increased on average during the mother-child interaction. Notably, this HRV profile is consistent with those seen in individuals with depression (104), as well as in those who are experiencing chronic stress (71, 105). Thus, our findings suggest a convergence of self-report and HRV in mothers who carry either full mutation or premutation at least in regards to depression. Given the relatively short time period of measurement and limited contexts of measurement in this study (i.e., 5 min during the baseline context and an average of 7 min during the mother-child interactions), it is not possible to discern whether the physiological findings are more indicative of traits or transient, situationally determined, states of stress. This should be explored further through the use of longer term HRV measurement across multiple contexts, as well as through other physiological indices of the ANS such as EDA and vagal tone. Moreover, the nature of such parent-child interactions (e.g., the presence of child challenging behaviors, preferred vs. non-preferred tasks, etc.) should be explored further to identify why they might elicit a greater stress response from these parents and how supports can most effectively be implemented. Moreover, although we removed motion artifacts from the data and both the baseline and dyadic interaction contexts entailed the mother being seated, it is possible that more subtle physical movements occurred in the two contexts. Future research should include additional control for movement as well as a similarly assessed and appropriately matched comparison group of mothers and their typically developing children to ensure that the differences in context we observed are, in fact, non-normative.

Although the HRV profiles observed in this study are consistent with what we found through parent self-report, exploratory analyses indicated no relationship between HRV and self-report indices of stress and well-being for the mothers in the present study (**Table 3**). This lack of relationship is, however, consistent with other studies of a similar nature looking at HRV in premutation carriers (75) and mothers of children with ASD (60) providing reason to believe that these two metrics capture different components of the individual's functioning and should, therefore, be further explored in tandem to gain a complete picture. Further, as with the findings for self-report measures of stress and well-being, mindfulness interventions impact HRV in positive ways as well (106, 107) and thus, could prove even more beneficial in populations such as mothers of children with FXS who display concerning profiles on both metrics of stress. Further, additional characteristics of the mother should be considered when implementing treatments. For instance, our finding regarding the relationship between depression and IQ might also suggest the need for different approaches to intervention among mothers of children with FXS.

Child Determinants of Maternal Well-Being

Lastly, we found considerable support for the role that the behavioral profile of the child with FXS, especially his or her level of externalizing behavior, plays in various aspects of maternal well-being; again, consistent with previous findings. Though limited by a small sample size and measurement at a single time point, which leaves the causal direction of the relationship unclear, the association between the two is consistent with the basic principles of the transactional model of development. In particular, the relationships are indicative that the child and

their environment are bidirectionally interconnected (108). Thus, in addition to supporting the mother as the individual, it is important to continue building upon prior work aimed at optimizing child outcomes, which could ultimately also have an added benefit for the well-being of the mother.

Limitations

This project is limited by a small sample size and thus many of our conclusions are considered preliminary and in need of further examination in a larger sample. This data set is also limited due to its lack of comprehensive information on genetic affectedness on behalf of the mothers beyond self-report of carrier status and thus future studies should explore more nuanced measures of genetic susceptibility to well-being not able to be examined here. Further, the mothers represented a relatively well-educated and resourced group and thus more work is needed to discern how our findings might generalize to a more under resourced sample. Also, given that data were collected at only one time point, future work would benefit from a more longitudinal approach to determine the bidirectional relationships of the associations we found. At the same time, the present sample of families was racially diverse, which may have been an outcome of our use of fully remote data collection procedures. These procedures may also have increased the representatives of the responses and behaviors of study participants who were able to complete the study in the familiar setting of the family home largely on a schedule that was maximally convenient for them. The inclusion of both self-report and physiological measures of stress was an additional positive and innovative feature of the study.

It is of note that our data collection was partially impacted by the COVID-19 pandemic, which contributed somewhat to recruitment challenges as well as contributing to one mother discontinuing early. Although in some instances, we observed increased availability of families due to being home, it also increased the level of stress and uncertainty per parent report during screening calls and they felt less inclined to add more burden to their own or their child's schedule by participating in a study, even one that did not require leaving home. Further, with regards to the data that were collected, 10 of the mother-child dyads completed the study prior to shelter-in place orders and the remaining 10 dyads completed data collection during the pandemic. We did assess potential differences on the dependent measures of maternal stress and well-being and child challenging behavior and found no differences between the two groups of mother-child dyads. These findings suggest that perhaps the families had adapted to conditions of the pandemic and were no more or less stressed than in the pandemic during pre-pandemic life, although it must be acknowledged that the small sample size and limited statistical power makes any conclusions tentative.

CONCLUSIONS AND FUTURE DIRECTIONS

Overall, the profiles of mental health status and parenting stress are reflective of what is seen in the larger literature as well as the relationship between child challenging behaviors and maternal well-being, which provides continued implications for the roll that supporting child development and behaviors can ultimately have on aspects of maternal well-being. Further, there were unique findings derived from this study including unique physiological profiles of stress and well-being that were separate from more standard measures (e.g., questionnaires) of stress and well-being in *FMR1* carriers. Moreover, a relative strength of this study is the use of multiple formats for measuring maternal well-being, including self-report as well as potentially more objective measures of well-being through the collection of maternal HRV. Another strength of the current study is the completion of the project entirely *via* distance through the use of video teleconferencing and online, electronically completed questionnaires. Further, implications across all of these findings support the need for dual support for both the mother and the child in order to achieve optimal outcomes. Despite the interconnectedness and importance of this relationship, however, there is a relative dearth of research on such combined interventions supporting multiple family members simultaneously.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Institutional Review Board at the University of California, Davis. All mothers provided electronic informed consent on behalf of themselves as participants and for their participating child prior to beginning data collection.

AUTHOR CONTRIBUTIONS

The submitted research was part of LB's dissertation research project. LB assisted with the design of the project and collected and analyzed all study data and wrote the corresponding manuscript. DH provided guidance on the statistical analysis plan. LA was the graduate advisor to LB and assisted her with the design of the project and oversaw data collection. All authors reviewed, edited, and approved this manuscript.

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REFERENCES

1. Borkowski JG, Ramey SL, Bristol-Power M, (Eds.). *Parenting and the Child's World: Influences on Academic, Intellectual, Social-Emotional Development*. Mahwah, NJ: Psychology Press (2001). doi: 10.4324/9781410603616
2. Landry SH, Smith KE, Swank PR. The importance of parenting during early childhood for school-age development. *Dev Neuropsychol.* (2003) 24:559–91. doi: 10.1080/87565641.2003.9651911
3. Siller M, Swanson M, Gerber A, Hutman T, Sigman M. A parent-mediated intervention that targets responsive parental behaviors increases attachment behaviors in children with ASD: results from a randomized clinical trial. *J Autism Dev Disord.* (2014) 44:1720–32. doi: 10.1007/s10803-014-2049-2
4. Van Hooste A, Maes B. Family factors in the early development of children with Down syndrome. *J Early Interv*. (2003) 25:296–309.
5. Hauser CT, Kover ST, Abbeduto L. Maternal well-being and child behavior in families with fragile X syndrome. *Res Dev Disabil.* (2014) 35:2477–86. doi: 10.1016/j.ridd.2014.06.012
6. Warren SF, Brady N, Sterling A, Fleming K, Marquis J. Maternal responsivity predicts language development in young children with fragile X syndrome. *Am J Intellectual Dev Disabil.* (2010) 115:54–75. doi: 10.1352/1944-7558-115.1.54
7. Hessl D, Dyer-Friedman J, Glaser B, Wisbeck J, Barajas RG, Taylor A, et al. The influence of environmental and genetic factors on behavior problems and autistic symptoms in boys and girls with fragile X syndrome. *Pediatrics*, (2001) 108:E88. doi: 10.1542/peds.108.5.e88
8. Abbeduto L, Seltzer MM, Shattuck P, Krauss MW, Orsmond G, Murphy MM. Psychological well-being and coping in mothers of youths with autism, down syndrome, or fragile X syndrome. *Am J Mental Retard.* (2004) 109:237–54. doi: 10.1352/0895-8017(2004)109<237:PWACIM>2.0.CO;2
9. Keenan BM, Newman LK, Gray KM, Rinehart, NJ. Parents of children with ASD experience more psychological distress, parenting stress, and attachment-related anxiety. *J Autism Dev Disord*. (2016) 46:2979–91. doi: 10.1007/s10803-016-2836-z
10. Wright AC, Taylor S. Advocacy by parents of young children with special needs: Activities, processes, perceived effectiveness. *J Soc Serv Res.* (2014) 40:591–605. doi: 10.1080/01488376.2014.896850
11. McAlindon A. *Preparing Those With Developmental Disabilities for Adulthood and Work: Parent Perspectives on Support Services*. Washington, DC (2018).
12. Karpur A, Vasudevan V, Lello A, Frazier TW, Shih A. Food insecurity in the households of children with autism spectrum disorders and intellectual disabilities in the United States: Analysis of the National Survey of Children's Health Data 2016-2018. *Autism.* (2021) 25:2400–11. doi: 10.1177/13623613211019159
13. Fielding-Gebhardt H, Warren SF, Brady NC. Child challenging behavior influences maternal mental health and relationship quality over time in Fragile X syndrome. *J Autism Dev Disord.* (2020) 50:779–97. doi: 10.1007/s10803-019-04308-2
14. Kennedy CH, Richman DM. Preventing challenging behaviors in people with neurodevelopmental disabilities. *Curr Dev Disord Rep.* (2019) 6:188–94. doi: 10.1007/s40474-019-00177-7
15. Haveman M, van Berkum G, Reijnders R, Heller T. Differences in service needs, time demands, and caregiving burden among parents of persons with mental retardation across the life cycle. *Family Relations.* (1997) 46:417–25. doi: 10.2307/585101
16. Weiss JA, Sullivan A, Diamond T. Parent stress and adaptive functioning of individuals with developmental disabilities. *J Dev Disabil.* (2003) 10:129–36.
17. Hall HR, Graff JC. The relationships among adaptive behaviors of children with autism, family support, parenting stress, and coping. *Issues Comprehen Pediatr Nursing.* (2011) 34:4–25. doi: 10.3109/01460862.2011.555270
18. Spain D, Sin J, Linder KB, McMahon J, Happé F. Social anxiety in autism spectrum disorder: A systematic review. *Res Autism Spect Disord.* (2018) 52:51–68. doi: 10.1016/j.rasd.2018.04.007
19. Roberts JE, Ezell JE, Fairchild AJ, Klusek J, Thurman AJ, McDuffie A, et al. Biobehavioral composite of social aspects of anxiety in young adults with fragile X syndrome contrasted to autism spectrum disorder. *Am J Med Genet Part B: Neuropsychiatric Genet.* (2018) 177:665–75. doi: 10.1002/ajmg.b.32674
20. Braden ML. Academic interventions. In: Hagerman RJ, Hagerman PJ, editors. *Fragile X, Syndrome: Diagnosis, Treatment, and Research*, Baltimore, MD: The Johns Hopkins University Press (2002). p. 423–464.
21. Miller LE, Burke JD, Troyb E, Knoch K, Herlihy LE, Fein DA. Preschool predictors of school-age academic achievement in autism spectrum disorder. *Clin Neuropsychol.* (2017) 31:382–403. doi: 10.1080/13854046.2016.1225665
22. Lewis P, Abbeduto L, Murphy M, Richmond E, Giles N, Bruno L, et al. Psychological well-being of mothers of youth with fragile X syndrome: Syndrome specificity and within-syndrome variability. *J Intellectual Disabil Res.* (2006) 50:894–904. doi: 10.1111/j.1365-2788.2006.00907.x
23. Allen EG, Charen K, Hipp HS, Shubeck L, Amin A, He W, et al. Clustering of comorbid conditions among women who carry an FMR1 premutation. *Genet Med.* (2020) 22:758–66. doi: 10.1038/s41436-019-0733-5
24. Hunter JE, Allen EG, Abramowitz A, Rusin M, Leslie M, Novak G, et al. Investigation of phenotypes associated with mood and anxiety among male and female fragile X premutation carriers. *Behav Genet.* (2008) 38:493–502. doi: 10.1007/s10519-008-9214-3
25. Hunter JE, Leslie M, Novak G, Hamilton D, Shubeck L, Charen K, et al. Depression and anxiety symptoms among women who carry the FMR1 premutation: impact of raising a child with fragile X syndrome is moderated by CRHR1 polymorphisms. *Am J Med Genet B Neuropsychiatr Genet*. (2012) 159:549–59. doi: 10.1002/ajmg.b.32061
26. Roberts JE, Bailey DB, Mankowski J, Ford A, Sideris J, Weisenfeld LA, et al. Mood and anxiety disorders in females with the FMR1 premutation. *Am J Med Genet B Neuropsychiatr Genet*. (2009) 150:130–9. doi: 10.1002/ajmg.b.30786
27. Belmonte MK, Bourgeron T. Fragile X syndrome and autism at the intersection of genetic and neural networks. *Nat Neurosci.* (2006) 9:1221–5. doi: 10.1038/nn1765
28. Hagerman RJ, Rivera SM, Hagerman PJ. The fragile X family of disorders: a model for autism and targeted treatments. *Curr Pediatr Rev.* (2008) 4:40–52. doi: 10.2174/157339608783565770
29. Nolin SL, Lewis FA, Ye LL, Houck Jr GE, Glicksman AE, Limprasert P, et al. Familial transmission of the FMR1 CGG repeat. *Am J Human Genet.* (1996) 59:1252.
30. Seltzer MM, Baker MW, Hong J, Maenner M, Greenberg J, Mandel D. Prevalence of CGG expansions of the FMR1 gene in a US population-based sample. *Am J Med Genet Part B: Neuropsychiatric Genet.* (2012) 159:589–97. doi: 10.1002/ajmg.b.32065
31. Garber KB, Visootsak J, Warren ST. Fragile X syndrome. *Eur J Human Genet.* (2008) 16:666–72. doi: 10.1038/ejhg.2008.61
32. Hagerman RJ, Jackson C, Amiri K, O'Connor R, Sobesky W, Silverman AC. Girls with fragile X syndrome: physical and neurocognitive status and outcome. *Pediatrics.* (1992) 89:395–400.
33. Gossett A, Sansone S, Schneider A, Johnston C, Hagerman R, Tassone F, et al. Psychiatric disorders among women with the fragile X premutation without children affected by fragile X syndrome. *Am J Med Genet Part B: Neuropsychiatric Genet.* (2016) 171:1139–47. doi: 10.1002/ajmg.b.32496
34. Debrey SM, Leehey MA, Klepitskaya O, Filley CM, Shah RC, Kluger B, et al. Clinical phenotype of adult fragile X gray zone allele carriers: a case series. *Cerebellum.* (2016) 15:623–31. doi: 10.1007/s12311-016-0809-6
35. Wheeler AC, Bailey DB Jr, Berry-Kravis E, Greenberg J, Losh M, Mailick M, Mila M, et al. Associated features in females with an FMR1 premutation. *J Neurodev Disord*. (2014) 6:30. doi: 10.1186/1866-1955-6-30
36. King G, King S, Rosenbaum P, Goffin R. Family-centered caregiving and well-being of parents of children with disabilities: linking process with outcome. *J Pediatr Psychol.* (1999) 24:41–53.
37. Franke P, Maier W, Hautzinger M, Weiffenbach O, Gänsicke M, Iwers B, et al. Fragile-X carrier females: Evidence for a distinct psychopathological phenotype?. *Am J Med Genet.* (1996) 64:334–9. doi: 10.1002/(SICI)1096-8628(19960809)64:2<334::AID-AJMG20>3.0.CO;2-F
38. Bailey DB, Raspa M, Olmsted M, Holiday DB. Co-occurring conditions associated with FMR1 gene variations: Findings from a national parent survey. *Am J Med Genetics Part A.* (2008) 146:2060–9. doi: 10.1002/ajmg.a.32439
39. Bourgeois JA, Seritan AL, Casillas EM, Hessl D, Schneider A, Yang Y, et al. Lifetime prevalence of mood and anxiety disorders in fragile X premutation carriers. *J Clin Psychiatry.* (2011) 72:175–82. doi: 10.4088/JCP.09m05407blu

40. Wheeler AC, Skinner DG, Bailey DB. Perceived quality of life in mothers of children with fragile X syndrome. *Am J Ment Retard AJMR*. (2008) 113:159–77.
41. Seltzer MM, Abbeduto L, Krauss MW, Greenberg J, Swe A. Comparison groups in autism family research: Down syndrome, fragile X syndrome, and schizophrenia. *J Autism Dev Disord*. (2004) 34:41–8. doi: 10.1023/B:JADD.0000018073.92982.64
42. Roberts JE, Tonnsen BL, McCary LM, Ford AL, Golden RN, Bailey, DB Jr. Trajectory and predictors of depression and anxiety disorders in mothers with the FMR1 premutation. *Biol Psychiatry*. (2016) 79:850–7. doi: 10.1016/j.biopsych.2015.07.015
43. Abbeduto L, Thurman AJ, McDuffie A, Klusek J, Feigles RT, Brown WT, et al. ASD comorbidity in fragile X syndrome: Symptom profile and predictors of symptom severity in adolescent and young adult males. *J Autism Dev Disord*. (2019) 49:960–77. doi: 10.1007/s10803-018-3796-2
44. Budimirovic DB, Kaufmann WE. What can we learn about autism from studying fragile X syndrome?. *Dev Neurosci*. (2011) 33:379–94. doi: 10.1159/000330213
45. McCary LM, Roberts JE. Early identification of autism in fragile X syndrome: a review. *J Intellect Disabil Res JIDR*. (2013) 57:803–14. doi: 10.1111/j.1365-2788.2012.01609.x
46. Smith LE, Barker ET, Seltzer MM, Abbeduto L, Greenberg JS. Behavioral phenotype of fragile X syndrome in adolescence and adulthood. *Am J Intellectual Dev Disabilities*. (2012) 117:1–17. doi: 10.1352/1944-7558-117.1.1
47. Oakes A, Thurman AJ, McDuffie A, Bullard LM, Hagerman RJ, Abbeduto L. Characterising repetitive behaviours in young boys with fragile X syndrome. *J Intellectual Disabil Res*. (2016) 60:54–67. doi: 10.1111/jir.12234
48. Wolff JJ, Hazlett HC, Lightbody AA, Reiss AL, Piven J. Repetitive and self-injurious behaviors: associations with caudate volume in autism and fragile X syndrome. *J Neurodev Disord*. (2013) 5:12. doi: 10.1186/1866-1955-5-12
49. Cordeiro L, Ballinger E, Hagerman R, Hessl D. Clinical assessment of DSM-IV anxiety disorders in fragile X syndrome: prevalence and characterization. *J Neurodev Disord*. (2011) 3:57–67. doi: 10.1007/s11689-010-9067-y
50. Lesniak-Karpiak K, Mazzocco MM, Ross JL. Behavioral assessment of social anxiety in females with Turner or fragile X syndrome. *J Autism Dev Disord*. (2003) 33:55–67. doi: 10.1023/A:1022230504787
51. Barker ET, Mailick MR, Smith LE. Chronic parenting stress in mothers of adolescents and adults with autism: Vulnerability and resilience. *Comprehen Guide Autism*. (2014) 4:207–22. doi: 10.1007/978-1-4614-4788-7_9
52. Reed GM, McLaughlin CJ, Milholland K. Ten interdisciplinary principles for professional practice in telehealth: Implications for psychology. *Professional Psychol Res Pract*. (2000) 31:170. doi: 10.1037/0735-7028.31.2.170
53. Lovallo WR. Can exaggerated stress reactivity and prolonged recovery predict negative health outcomes? The case of cardiovascular disease. *Psychosom Med*. (2015) 77:212–4. doi: 10.1097/PSY.0000000000000173
54. Coffey SM, Cook K, Tartaglia N, Tassone F, Nguyen DV, Pan R, et al. Expanded clinical phenotype of women with the FMR1 premutation. *Am J Med Genet Part A*. (2008) 146A:1009–16. doi: 10.1002/ajmg.a.32060
55. Johnston C, Hessl D, Blasey C, Eliez S, Erba H, Dyer-Friedman J, et al. Factors associated with parenting stress in mothers of children with fragile X syndrome. *J Dev Behav Pediatr JDBP*. (2003) 24:267–75. doi: 10.1097/00004703-200308000-00008
56. Grebner S, Elfering A, Semmer N. K, Kaiser-Probst C, Schlapbach ML. Stressful situations at work and in private life among young workers: an event sampling approach. *Soc Indic Res*. (2004) 67:11–49.
57. Seltzer MM, Greenberg JS, Hong J, Smith LE, Almeida DM, Coe C, et al. Maternal cortisol levels and behavior problems in adolescents and adults with ASD. *J Autism Dev Disord*. (2010) 40:457–69. doi: 10.1007/s10803-009-0887-0
58. Wong JD, Seltzer MM, Greenberg JS, Hong J, Almeida DM, Coe CL. Stressful life events and daily stressors affect awakening cortisol level in midlife mothers of individuals with autism spectrum disorders. *Aging Mental Health*. (2012) 16:939–49. doi: 10.1080/13607863.2012.688191
59. Ruiz-Robledillo N, Moya-Albiol L. Lower electrodermal activity to acute stress in caregivers of people with autism spectrum disorder: An adaptive habituation to stress. *J Autism Dev Disord*. (2015) 45:576–88. doi: 10.1007/s10803-013-1996-3
60. Factor RS, Swain DM, Scarpa A. Child autism spectrum disorder traits and parenting stress: The utility of using a physiological measure of parental stress. *J Autism Dev Disord*. (2018) 48:1081–91. doi: 10.1007/s10803-017-3397-5
61. Padden C, James JE. Stress among parents of children with and without autism spectrum disorder: a comparison involving physiological indicators and parent self-reports. *J Dev Phys Disabil*. (2017) 29:567–86. doi: 10.1007/s10882-017-9547-z
62. Robinson AN, Roberts JE, Brady NC, McQuillin SD, Warren SF. Physiological correlates of maternal responsivity in mothers of preschoolers with fragile X syndrome. *Am J Intellect Dev Disabil*. (2016) 121:111–20. doi: 10.1352/1944-7558-121.2.111
63. Hartley SL, Seltzer MM, Hong J, Greenberg JS, Smith L, Almeida D, et al. Cortisol response to behavior problems in FMR1 premutation mothers of adolescents and adults with fragile X syndrome: A diathesis-stress model. *Int J Behav Dev*. (2012) 36:53–61. doi: 10.1177/0165025411406857
64. Kreibig SD. Autonomic nervous system activity in emotion: a review. *Biol Psychol*. (2010) 84:394–421. doi: 10.1016/j.biopsycho.2010.03.010
65. Ulrich-Lai YM, Herman JP. Neural regulation of endocrine and autonomic stress responses. *Nat Rev Neurosci*. (2009) 10:397–409. doi: 10.1038/nrn2647
66. Appelhans BM, Luecken LJ. Heart rate variability as an index of regulated emotional responding. *Rev Gene Psychol*. (2006) 10:229–40. doi: 10.1037/1089-2680.10.3.229
67. Yaroslavsky I, Rottenberg J, Kovacs M. Atypical patterns of respiratory sinus arrhythmia index an endophenotype for depression. *Dev Psychopathol*. (2014) 26(4 Pt 2):1337–52. doi: 10.1017/S0954579414001060
68. Thayer JF, Brosschot JF. Psychosomatics and psychopathology: looking up and down from the brain. *Psychoneuroendocrinology*. (2005) 30:1050–8. doi: 10.1016/j.psyneuen.2005.04.014
69. Hamilton JL, Alloy LB. Atypical reactivity of heart rate variability to stress and depression across development: Systematic review of the literature and directions for future research. *Clin Psychol Rev*. (2016) 50:67–79. doi: 10.1016/j.cpr.2016.09.003
70. Shaffer F, McCraty R, Zerr CL. A healthy heart is not a metronome: an integrative review of the heart's anatomy and heart rate variability. *Front Psychol*. (2014) 5:1040. doi: 10.3389/fpsyg.2014.01040
71. Romero-Martínez Á, Moya-Albiol L. Reduced cardiovascular activation following chronic stress in caregivers of people with anorexia nervosa. *Stress*. (2017) 20:390–7. doi: 10.1080/10253890.2017.1343815
72. Kim HG, Cheon EJ, Bai DS, Lee YH, Koo BH. Stress and heart rate variability: a meta-analysis and review of the literature. *Psychiatry Invest*. (2018) 15:235–45. doi: 10.30773/pi.2017.08.17
73. Held J, Vîslă A, Wolfer C, Messerli-Bürgy N, Flückiger C. Heart rate variability change during a stressful cognitive task in individuals with anxiety and control participants. *BMC Psychol*. (2021) 9:1–8. doi: 10.1186/s40359-021-00551-4
74. Tolin DF, Lee E, Levy HC, Das A, Mammo L, Katz BW, et al. Psychophysiological assessment of stress reactivity and recovery in anxiety disorders. *J Anxiety Disord*. (2021) 82:102426. doi: 10.1016/j.janxdis.2021.102426
75. Klusek J, LaFauci G, Adayev T, Brown WT, Tassone F, Roberts JE. Reduced vagal tone in women with the FMR1 premutation is associated with FMR1 mRNA but not depression or anxiety. *J Neurodev Disord*. (2017) 9:1–16. doi: 10.1186/s11689-017-9197-6
76. Woodman AC. Trajectories of stress among parents of children with disabilities: A dyadic analysis. *Family Relations*. (2014) 63:39–54. doi: 10.1111/fare.12049
77. Orr R, Cameron SJ, Dobson LA, Day DM. Age-related changes in stress experienced by families with a child who has developmental delays. *Mental Retardation*. (1993) 31:171–6.
78. Naglieri JA, Bardos AN. General Ability Measure for Adults. Minneapolis, MN: National Computer Systems (1997).
79. Sparrow SS, Cicchetti DV, Saulnier CA. Vineland-3: Vineland Adaptive Behavior Scales. *PsychCorp*. (2016).
80. Schopler E, Reichler RJ, Renner BR. *The Childhood Autism Rating Scale (CARS)*. Los Angeles, CA: WPS (2010).
81. Achenbach TM. *Achenbach System of Empirically Based Assessment: School Age Forms and Profiles, Child Behavior Checklist for Ages*. (2001) 6–18.

82. Derogatis LR. SCL-90-R. *Symptom Checklist-90-R*. Instructions for administration and scoring. (2009).
83. Abidin RR. *Parenting Stress Index-Fourth Edition (PSI-4)*. Lutz, FL: Psychological Assessment Resources. (2012).
84. McCarthy C, Pradhan N, Redpath C, Adler A. Validation of the empatica E4 wristband. In: *2016 IEEE EMBS International Student Conference (ISC)*, (2016) 1–4. doi: 10.1109/EMBSISC.2016.7508621
85. Schuurmans A, de Looff P, Nijhof KS, Rosada C, Scholte R, Popma A, et al. Validity of the empatica E4 wristband to measure heart rate variability (HRV) parameters: a comparison to electrocardiography (ECG). *J Med Sys*. (2020) 44:190. doi: 10.1007/s10916-020-01648-w
86. Shaffer F, Ginsberg JP. An overview of heart rate variability metrics and norms. *Front Public Health*. (2017) 5:258. doi: 10.3389/fpubh.2017.00258
87. Brody DJ, Pratt LA, Hughes J. *Prevalence of Depression Among Adults Aged 20 and Over: United States, 2013–2016. NCHS Data Brief, no 303*. Hyattsville, MD: National Center for Health Statistics (2018).
88. Harvard Medical School. *National Comorbidity Survey (NCS)*. (2007). Available online at: https://www.hcp.med.harvard.edu/ncs/index.php. Data Table 2: 12-month prevalence DSM-IV/WMH-CIDI disorders by sex and cohort (accessed August 21, 2017).
89. Agorastos A, Stiedl O, Heinig A, Sommer A, Hager T, Freundlieb N, et al. Inverse autonomic stress reactivity in depressed patients with and without prior history of depression. *J Psychiatric Res.* (2020) 131:114–8. doi: 10.1016/j.jpsychires.2020.09.016
90. Wekenborg MK, von Dawans B, Hill LK, Thayer JF, Penz M, Kirschbaum C. Examining reactivity patterns in burnout and other indicators of chronic stress. *Psychoneuroendocrinology*. (2019) 106:195–205. doi: 10.1016/j.psyneuen.2019.04.002
91. Nunan D, Sandercock GR, Brodie DA. A quantitative systematic review of normal values for short-term heart rate variability in healthy adults. *Pacing Clin*. Electrophysiol PACE (2010) 33:1407–17. doi: 10.1111/j.1540-8159.2010.02841.x
92. Long ACJ. *Parenting Stress and Social Support in Families With Children With Fragile X Syndrome: A Comparison of Mothers and Fathers* (Doctoral dissertation), The University of North Carolina at Chapel Hill (2009).
93. Izadi-Mazidi M, Riahi F, Khajeddin N. Effect of cognitive behavior group therapy on parenting stress in mothers of children with autism. *Iran J Psychiatry Behav Sci.* (2015) 9:1900. doi: 10.17795/ijpbs-1900
94. Harper A, Dyches TT, Harper J, Roper SO, South M. Respite care, marital quality, and stress in parents of children with autism spectrum disorders. *J Autism Dev Disord.* (2013) 43:2604–16. doi: 10.1007/s10803-013-1812-0
95. Craig F, Operto FF, De Giacomo A, Margari L, Frolli A, Conson M, et al. Parenting stress among parents of children with neurodevelopmental disorders. *Psychiatry Res.* (2016) 242:121–9. doi: 10.1016/j.psychres.2016.05.016
96. Hartley SL, Wheeler AC, Mailick MR, Raspa M, Mihaila I, Bishop E, et al. Autism symptoms across adulthood in men with fragile X syndrome: a cross-sectional analysis. *J Autism Dev Disord.* (2015) 45:3668–79. doi: 10.1007/s10803-015-2513-7
97. Miodrag N, Hodapp RM. Chronic stress and health among parents of children with intellectual and developmental disabilities. *Curr Opin Psychiatry.* (2010) 23:407–11. doi: 10.1097/YCO.0b013e32833a8796
98. Shapiro SL, Brown KW, Biegel GM. Teaching self-care to caregivers: Effects of mindfulness-based stress reduction on the mental health of therapists in training. *Train Educ Profess Psychol.* (2007) 1:105. doi: 10.1037/1931-3918.1.2.105
99. Kautz C, Parr J, Petrenko CL. Self-care in caregivers of children with FASD: How do caregivers care for themselves, and what are the benefits and obstacles for doing so?. *Res Dev Disabil.* (2020) 99:103578. doi: 10.1016/j.ridd.2020.103578
100. Hunter JE, Jenkins CL, Grim V, Leung S, Charen KH, Hamilton DR, et al. Feasibility of an app-based mindfulness intervention among women with an FMR1 premutation experiencing maternal stress. *Res Dev Disabil*. (2019) 89:76–82. doi: 10.1016/j.ridd.2019.03.008
101. Wheeler AC, Miller S, Wylie A, Edwards A. Mindfulness and acceptance as potential protective factors for mothers of children with fragile X syndrome. *Front Public Health.* (2018) 6:316. doi: 10.3389/fpubh.2018.00316
102. Hall SS, Monlux KD, Rodriguez AB, Jo B, Pollard JS. Telehealth-enabled behavioral treatment for problem behaviors in boys with fragile X syndrome: a randomized controlled trial. *J Neurodev Disord.* (2020) 12:31. doi: 10.1186/s11689-020-09331-4
103. Stahmer AC, Pellecchia M. Moving towards a more ecologically valid model of parent-implemented interventions in autism. *Autism*. (2015) 19:259–61. doi: 10.1177/1362361314566739
104. Rottenberg J. Cardiac vagal control in depression: a critical analysis. *Biol Psychol*. (2007) 74:200–11. doi: 10.1016/j.biopsycho.2005.08.010
105. Schubert C, Lambertz M, Nelesen RA, Bardwell W, Choi JB, Dimsdale JE. Effects of stress on heart rate complexity-a comparison between short-term and chronic stress. *Biol Psychol.* (2009) 80:325–32. doi: 10.1016/j.biopsycho.2008.11.005
106. Shearer A, Hunt M, Chowdhury M, Nicol L. Effects of a brief mindfulness meditation intervention on student stress and heart rate variability. *Int J Stress Manage.* (2016) 23:232. doi: 10.1037/a0039814
107. Mankus AM, Aldao A, Kerns C, Mayville EW, Mennin DS. Mindfulness and heart rate variability in individuals with high and low generalized anxiety symptoms. *Behav Res Therapy.* (2013) 51:386–91. doi: 10.1016/j.brat.2013.03.005
108. Sameroff A. *The Transactional Model*. Washington, DC: American Psychological Association (2009). doi: 10.1037/11877-001

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Neural Correlates of Infant Face Processing and Later Emerging Autism Symptoms in Fragile X Syndrome

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Fragile X syndrome (FXS) is the leading known genetic cause of autism spectrum disorder (ASD) with 60–74% of males with FXS meeting diagnostic criteria for ASD. Infants with FXS have demonstrated atypical neural responses during face processing that are unique from both typically developing, low-risk infants and infants at high familial risk for ASD (i.e., infants siblings of children with ASD). In the current study, event-related potential (ERP) responses during face processing measured at 12 months of age were examined in relation to ASD symptoms measured at ∼48 months of age in participants with FXS, as well as siblings of children with ASD and low-risk control participants. Results revealed that greater amplitude N290 responses in infancy were associated with more severe ASD symptoms in childhood in FXS and in siblings of children with ASD. This pattern of results was not observed for low-risk control participants. Reduced Nc amplitude was associated with more severe ASD symptoms in participants with FXS but was not observed in the other groups. This is the first study to examine ASD symptoms in childhood in relation to infant ERP responses in FXS. Results indicate that infant ERP responses may be predictive of later symptoms of ASD in FXS and the presence of both common and unique pathways to ASD in etiologically-distinct high-risk groups is supported (i.e., syndromic risk vs. familial risk).

Keywords: event-related potentials, fragile X syndrome, autism spectrum disorder, infant, child

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INTRODUCTION

Fragile X syndrome (FXS) is a single-gene disorder that results from a CGG repeat expansion mutation on the X chromosome affecting approximately one in 3,700–8,900 males (1–4) and one in 11,100 females (4). FXS possesses a high level of comorbidity with autism spectrum disorder (ASD) and is the most common single-gene cause of ASD, evidenced by 60–74% of individuals with FXS meeting diagnostic criteria for ASD (5–9). This is much higher than the rate of 1.9%, which is observed in the general population (10). Research has increasingly focused on early detection of ASD in FXS with evidence suggesting that 61% of preschool children meet diagnostic criteria with a high degree of diagnostic certainty (9). Likewise, behavioral risk markers for ASD are present in infants with FXS by 12 months of age (11, 12) and are predictive of later ASD diagnoses (9). In addition, specific neural responses [i.e., event-related potentials (ERPs)] related to ASD are also atypical in infants with FXS at 12 months of age (13). However, the relationship between neural

responses in infancy and ASD symptom severity in early childhood have not been reported as we do in the current study. Research on the early development of ASD symptoms in infants and children with FXS provides insight into multiple developmental pathways to ASD, which may improve identification of reliable risk markers in infancy and facilitate earlier diagnosis and intervention.

To date, nearly all of the research examining early risk markers of ASD has been conducted on infant siblings of children diagnosed with ASD (henceforth referred to as ASIBs), who are at elevated risk for ASD because of the significant heritability of ASD. Approximately 20% of ASIBs will be diagnosed with ASD themselves (14–16), and another 20–40% will exhibit other developmental differences (14). A review of literature on the development of ASD in ASIBs found that the first behavioral signs of ASD typically emerged between 6 and 18 months of age and were most frequently associated with atypical patterns of social attention (16). Other behavioral studies have shown that infants at high risk for ASD demonstrate decreased attention to social stimuli, including faces [e.g., (17, 18)]. In one study, this was reported in infants as young as 1 week old (19). Still, there are additional studies that do not report early atypical social attention in high risk infants [e.g., (20, 21)].

Recent work focused on understanding the early signs of ASD in infants with FXS suggests that those infants with FXS that are later diagnosed with ASD demonstrate early social-communicative deficits similar to those observed in ASIBs later diagnosed with ASD (9, 11). In a series of case studies, Hogan and colleagues (11) followed eight infants with FXS longitudinally from 9 to 24 months of age. They found that the four infants with FXS that were later diagnosed with ASD demonstrated impairments in social communication, including reduced social interest, social smiling, and babbling, which were not consistently displayed in the four infants that were not later diagnosed with ASD. Additional work has shown that atypical eye contact (9) and social avoidance (22) in infancy are also associated with greater severity of ASD symptoms in young children with FXS. Although not necessarily social in nature, atypical patterns of visual attention (23) and physiological arousal (9, 24) have also been related to the presentation of symptoms of ASD in infants with FXS.

Despite significant progress in understanding how behavioral symptoms of ASD emerge and change across early development (16), much remains to be learned about heterogeneity in the early development of ASD and the presentation of reliable risk markers in the first year of life. As subsets of both infants with FXS and ASIBs show behavioral ASD symptoms that emerge in infancy and are predictive of later diagnoses of ASD, examining early neural risk markers in these groups from a cross-syndrome approach may lead to an increased understanding of each group individually, as well as inform understanding of heterogeneous pathways to ASD (25). Recent work indicates that atypical infant brain responses are early-appearing and reliable indicators of ASD risk in ASIBs (26). Specifically, atypical patterns of neural activation during face processing have been reported in studies of infants with FXS (13) and ASIBs [e.g., (13, 27–30)]. Benefits of an ERP approach to investigating risk markers for ASD are seen in their ability to detect unique patterns of brain activity, which may emerge prior to behaviors associated with ASD and may be evident in an infant sample that is inherently limited by a restricted range of behaviors. Additionally, ERP measures may provide a more objective measure of risk than assessment of behavior. In the current study, we investigated the possibility that neural responses to social (i.e., face) and non-social (i.e., toy) stimuli in infancy, measured through ERPs, may be associated with later-emerging symptoms of ASD in infants with FXS contrasted against etiologically-distinct high-risk infants (ASIBs) and low-risk infants.

In the first months of life, face processing is believed to occur within a subcortical pathway that is recruited to a lesser extent with age as cortical pathways become specialized for face processing (31, 32). If early visual attention to social stimuli is similar across high- and low-risk infants, it may be because they rely on the same, intact subcortical pathway (31). From this perspective, deficits are expected to emerge near the end of the first year of life, as cortical pathways become established, and show atypical function associated with emerging ASD (31, 33). Further evidence that the timing of cortical pathways influence social attention near the end of the first year of life comes from behavioral work recently described by Ozonoff and Iosif (34), indicating that a regression in social attention (e.g., eye contact) from 6 to 12 months of age was present in 86% of ASIBs that went on to receive a positive diagnosis for ASD. They report that group differences in social attention are rarely seen before 9 months of age, but seem to emerge around 12 months and then increase in magnitude with age. Taking this evidence into account, it has been posited that deficits in the function of the "social brain" may become evident earlier in development at the neural level than at the behavioral level. ERPs show excellent sensitivity to neural timing and patterns of stimulus responses and have the potential to show high sensitivity for identifying atypical responses to stimuli, such as faces, in infancy, before behavioral symptoms of ASD manifest. As such, the use of ERPs in research with high-risk infants may allow for the identification of an early, reliable marker associated with the later emergence of ASD.

In typically developing infants, the N290 and P400 ERP components have been identified as possible precursors to the N170 ERP component, which is associated with face specialization in adults [e.g., (29)]. The N290 peaks ∼290 ms after stimulus onset and is most similar to the N170, as they are both negative peaks observed at lateral posterior electrode sites [e.g., (35, 36)]. Like the N170, the N290 is greater in amplitude in response to faces than other stimuli (35, 37). The P400 is a positive amplitude ERP component that peaks at ∼400 ms after stimulus onset over occipital scalp sites (35, 36, 38). The role of the P400 in social information processing in infancy is less well understood. For example, some studies have reported shorter latency to faces than other stimuli (30, 37, 39), others have reported greater amplitude to non-face than face stimuli (36), and others have found no significant effects based on stimulus category (13, 35).

The N290 and P400 have also been investigated in infant ASIBs, although the effect of risk on neural correlates of face

processing is not straightforward. Some studies have found greater amplitude N290 response to faces than other classes of stimuli in ASIBs (13, 30). Luyster and colleagues (29) conducted a large-scale longitudinal study of ERP components in 61 ASIBs and 70 low-risk control (LRC) infants from 6 to 36 months of age. Their results indicated similar developmental trajectories of the N290 and P400 across groups, and included only marginally significant group differences in N290 responses to the infant's mother's face vs. a stranger's face. They found that LRC infants demonstrated greater differentiation of these stimulus categories than ASIBs. Additionally, there was a marginally significant interaction of participant group and stimulus category on P400 amplitude. Nine ASIB participants later received an ASD diagnosis, however, the authors reported that inclusion or exclusion of participants based on ASD diagnosis did not significantly impact the results.

An additional ERP component that is of great interest in infant research is the Negative central (Nc), which occurs ∼350–750 ms after stimulus onset at midline frontal and central electrodes (40). The Nc is not directly associated with social processing but is indicative of attentional engagement and is observed in response to a wide range of visual stimuli. Nc amplitude is typically greater in response to novel or salient stimuli than familiar stimuli (39, 41–44). Studies measuring infant heart rate responses have also found that Nc amplitude is greater during heart rate-defined periods of sustained attention (36, 45). The Nc is of interest to the current investigation, as it may provide insight into the presence of atypical attentional allocation in infancy, which would be expected to reflect more general processing deficits, less closely associated with social information processing specifically.

Guy and colleagues (13) conducted the first investigation of neural correlates of face processing in multiple groups of infants at high risk for ASD, including infants with FXS and ASIBs. ERPs were measured in response to familiar and novel faces and toys. Across participant groups, a greater amplitude N290 was observed to faces than toys. Differences in N290 amplitude to faces and toys were most pronounced in infants with FXS and smallest in ASIBs. Additionally, visual examination of the data revealed that infants with FXS showed an enhanced N290 response relative to the other two participant groups. This was reflected in a significant group by stimulus familiarity interaction. Infants with FXS showed greater N290 amplitude to familiar stimuli than novel stimuli, while other participants did not discriminate stimuli based on familiarity at the N290. Furthermore, responses to familiar stimuli in infants with FXS were greater than ASIBs' and LRC infants' responses to familiar and novel stimuli. No significant differences were observed for the P400 across group or stimulus type. At the Nc ERP component, ASIBs demonstrated a more muted response than infants with FXS and LRC infants. Although Nc amplitude did not differ across face and toy stimuli, there was an effect of stimulus familiarity on Nc responses. LRCs showed a greater Nc response to novel (i.e., a stranger's face, a novel toy) than familiar stimuli (i.e., their mother's face, a favorite toy). Infants with FXS showed a greater Nc response to familiar stimuli as opposed to novel stimuli, which has been observed in some research conducted with younger (i.e., 6-month-old) infants with typical development (39, 41, 43). Interestingly, ASIBs did not differentiate stimuli based on familiarity.

These results indicate that while both infants with FXS and ASIBs are at an increased risk of developing ASD, differing patterns of neural responses to social and non-social stimuli are observed across these two etiologically-distinct high-risk groups. Not only do their ERP responses differentiate them from low-risk control infants, but also from each other. What remains to be known is whether group differences in N290 amplitude responses in infants with FXS and ASIBs may be associated specifically with emerging symptoms of ASD. The enhanced N290 in infants with FXS could reflect a hyperactive or hypervigilant neural response to social stimuli that could be related to later social anxiety, which is highly prevalent in FXS [e.g., (46)]. Furthermore, a more muted response in ASIBs may reflect a reduced, hypoactive response indicating reduced social interest and salience, as has been observed in individuals with ASD [for review see (47)].

The objective of the current study was to determine how the neural correlates of faces processing in infancy relate to ASD symptoms later in childhood in children with FXS compared with another group at high risk for ASD (i.e., ASIBs) and LRC children. In the current study, we utilized previously collected ERP data from high-risk infants and LRCs (13) to examine the relations between infant ERP responses during a face processing task and ASD symptom severity in early childhood. Infant ERP responses were measured at 12 months of age and clinical assessment for ASD was conducted later in childhood, at ∼48 months of age. In an approach similar to that used by Elsabbagh and colleagues (48), relations between N290, P400, and Nc amplitude and continuous scores of ASD symptom severity were investigated. Severity scores were used to quantify overall ASD symptoms, social symptoms, and restricted and repetitive behavior symptoms. We hypothesized that N290 responses to faces would be associated with overall symptom scores, as well as social scores, but that the pattern of relations would vary across high-risk groups. Specifically, we expected that enhanced N290 amplitude in infants with FXS would be related to higher ASD symptom scores, while muted N290 amplitude in ASIBs would be associated with higher ASD symptom scores. As the N290 is uniquely sensitive to face stimuli, we did not believe that N290 responses would be closely linked to restricted and repetitive behavior scores. Additionally, based on the hypothesized role of the P400 in social information processing, we predicted that the P400 may be associated with overall or social affect symptom scores, but that it would be less closely correlated with restricted and repetitive behavior scores. However, due to the observation of similar P400 responses across infants with FXS, ASIBs, and LRCs in previous research (13), we expected that the P400 would be less likely to be significantly related to ASD symptoms than the N290. Differences were previously observed across groups in Nc responses, but based on the Nc's responsiveness to a wide range of visual stimuli, we believed that the Nc may be more broadly associated with overall ASD scores and not linked with a specific domain of ASD symptoms. For example, relations between Nc responses and symptoms of ASD were not expected to be face specific, and to have connections to symptom severity scores across the three different scales. We examined relations across

face and toy and familiar and novel stimuli, based on significant effects observed in this group in infancy (13).

METHODS

Participants

Fifty 12-month-old infants were included in the study, including 14 infants with FXS (seven males), 18 ASIBs (15 males), and 18 typically developing low-risk control (LRC) infants (14 males). All participants were retained from our previous research study (13). An additional infant with FXS, three ASIBs, and three LRC infants were tested in the original Guy et al. (13) study, but were not retained in the current study due to lack of outcome data. Infants with FXS were identified through collaborations with researchers across the United States in addition to emails and postings through social media. ASIBs were recruited through the South Carolina Department of Disabilities and Special Needs; a letter was sent to families with a child with an ASD diagnosis, inviting participation from families with an infant sibling. LRC infants were recruited from the Columbia, SC area and were required to have no known developmental anomalies and no family history of ASD or related disorders (e.g., FXS). Participants were recruited without regard to race, ethnicity, socioeconomic status, and gender. However, participants were primarily Caucasian and of middle socioeconomic status. All infants participated with the informed, signed consent of their parents.

Measures and Apparatus

EEG (Infant Timepoint)

EEG was recorded in 12-month-olds using the Electrical Geodesics, Inc. (EGI) high-density 128-channel EEG system. Participants were seated on a parent's lap during the recording. They were positioned about 55 cm from a 29″ LCD monitor (NEC Multisync XM29). A video camera was just above the monitor and used to record participant looking behavior. An experimenter judged infant fixation online and controlled stimulus presentation using EGI Net Station and E-Prime software in an adjacent room. Stimuli included photographs of female faces (i.e., the mother's face and a stranger's face) and infant toys (i.e., a picture of a toy belonging to the infant and a novel toy). Sesame Street video clips were used as attractors when children lost interest in the stimuli. All stimuli were presented on colorful, variegated backgrounds [see (13) for more details].

ASD Symptoms (Outcome Timepoint)

Participants were followed longitudinally as part of a larger study on development in high-risk infants, and ASD symptoms were assessed annually at outcome timepoints beginning at 24 months of age. For the current study, we targeted clinical data from their 36-month visit or later, as ASD symptoms and diagnoses assessed at this age appear to be stable (9, 49, 50). Data from the 24-month visit were used if no later data were available ($n = 0$ children with FXS, $n = 2$ ASIBs, $n = 2$ LRC children). Although assessment of ASD symptoms prior to 3 years of age are generally considered less reliable, recent research has provided promising evidence of symptom and diagnostic stability starting at 18 months (51–53).

TABLE 1 | ADOS calibrated severity scores by participant group.

	ASIB	FXS	LRC
n	18	14	18
n male (%)	15 (83%)	7 (50%)	14 (78%)
Age in months at ADOS	48.00 (20.56)	47.14 (9.94)	44.67 (14.73)
Overall CSS (*SD*)	4.06 (3.00)	4.79 (2.46)	2.28 (1.78)
SA CSS (*SD*)	4.22 (2.73)	4.43 (2.77)	2.61 (1.94)
RB CSS (*SD*)	5.11 (3.31)	5.93 (3.17)	2.94 (2.62)

The mean age of outcome assessment was similar across groups (ASIB: $M = 48.00$ months, FXS: $M = 47.14$ months, LRC: $M = 44.67$ months).

The Autism Diagnostic Observation Schedule—2nd Edition [ADOS-2; (54)] was used to measure ASD symptoms. Overall, Social Affect (SA), and Restricted and Repetitive Behavior (RRB) calibrated severity scores (CSS) were computed using established guidelines (55, 56). The CSS has been established as a stable continuous measure of ASD severity that is more valid than the overall ADOS raw score (57). Average calibrated severity scores are presented by group in **Table 1**.

Procedure

EEG

Participants were fitted with an EGI "hydrocel geodesic sensor net" (HGSN) that was selected based on their head circumference. Net application took 5–10 min, during which a second experimenter entertained the infant with toys. The experiment commenced once the infant was positioned in front of the monitor. An attractor stimulus was used to draw fixation toward the center of the screen and a button was used to indicate fixation and to begin stimulus presentation, which included brief stimulus presentations and visual paired comparison (VPC) trials. Brief stimulus presentations included a 100 ms blank screen baseline period, followed by a 500 ms stimulus presentation, and a variable inter-trial interval of 500–1500 ms. The VPC trials included side-by-side presentations of the two face stimuli or the two toy stimuli and lasted until 4 s of looking time was reached. The VPC and brief stimulus presentations were presented in random order in 10-trial blocks. If the infant looked away from the screen, the Sesame Street attractor stimulus was used to regain fixation toward the screen. Stimulus presentation continued until the infant became bored or fussy.

Data Analysis

EEG Recording and Analysis

The EEG was recorded from 124 electrodes in the EGI HGSN, two additional electrodes measured electrooculogram (EOG), and two electrodes measured electrocardiogram (ECG). Recordings were referenced to the vertex online, recorded with 20K amplification at a 250 Hz sampling rate with bandpass filters set from 0.1 to 100 Hz and 100 kΩ impedance. Data processing was completed using the EEGLAB and ERPLAB

Matlab toolboxes (58, 59). Following data collection, the vertex-referenced EEG was algebraically recomputed to the average reference. The EEG was filtered with a 0.5 Hz high-pass filter and ERP trials were segmented from 50 ms before stimulus onset through 1 s following onset. Recorded EEG was inspected for artifact (defined as a change in amplitude >100 μV), poor recordings, and blinks using the ERPLAB toolbox in Matlab and visual inspection. Trials were eliminated from further analyses if more than 10 channels were affected.

Clusters of virtual "10-10" electrodes were created from the mean of the EGI electrodes surrounding the traditional 10-10 electrode locations [see (36) Supplemental Information]. The N290 was examined at lateral posterior-inferior electrodes including Parietal Occipital (PO7: 59, 65, 66; PO8: 84, 90, 91; PO9: 64, 65, 68, 69; PO10: 89, 90, 94, 95), Parietal (P7: 51, 58, 59; P8: 91, 96, 97; P9: 57, 58, 63, 64; P10: 95, 96, 99, 100), and Temporal Parietal electrodes (TP7: 46, 50, 51; TP8: 97, 101, 102; TP9: 50, 56, 57; TP10: 100, 101, 107). The P400 was examined at medial posterior-inferior electrodes including Parietal Occipital (PO7–10), Occipital (Oz: 71, 75, 76; O1: 66, 70, 71; O2: 76, 83, 84), and Inion electrodes (Iz: 74, 75, 81, 82; I1: 69, 70, 73, 74; I2: 82, 83, 88, 89). The Nc was analyzed at frontal and central midline virtual electrodes (Fz: 5, 10, 11, 12, 16, 18; FCz: 5, 6, 7, 12, 106; Cz: 7, 31, 55, 80, 106). Additional information on the selection of time windows for ERP component analysis and computation of ERP amplitude can be found in our previous publications (13, 35, 60).

To better understand relations between early neural responses to visual social and non-social stimuli and later symptoms of ASD, we assessed ADOS-2 calibrated severity scores in relation to infant ERP responses. In extension of methods utilized in past research (13), amplitude of the N290, P400, and Nc ERP components based on stimulus type (2: faces, toys) and stimulus familiarity (2: familiar, novel) were examined in association with Overall, Social Affect (SA), and Restricted and Repetitive Behavior (RRB) CSS using ANCOVAs and regressions. A general linear models approach (i.e., "Proc GLM" of SAS) using nonorthogonal design was used. The statistical tests used error terms derived from the related interval effect analyses to control for inflation of test wise error rate. All significant tests are reported at $p < 0.05$ and effect sizes (eta squared) and 95% confidence intervals for effect sizes are reported to describe comparisons within significant effects.

RESULTS

N290

Graphs presenting the relations between N290 amplitude to faces and toys across infants with FXS, ASIBs, and LRC infants and Overall, Social Affect, and Restricted and Repetitive Behavior CSS on the ADOS-2 are presented in **Figure 1**.

Overall CSS

There was a significant interaction between participant group, stimulus type, and Overall CSS on N290 amplitude, $F_{(2, 1188)} = 10.05$, $p < 0.001$, $n_p^2 = 0.02$, 95% CI [0.01, 0.03]. To better understand this interaction, we examined responses to faces and toys separately. In response to faces, there was a significant interaction of Overall CSS and group, $F_{(2, 594)} = 15.65$, $p < 0.001$, $n_p^2 = 0.05$, 95% CI [0.02, 0.09]. For infants with FXS and ASIBs, a more negative amplitude N290 in response to faces was associated with higher Overall CSS (infants with FXS: $F_{(1, 166)} = 7.72$, $p = 0.006$, $n_p^2 = 0.04$, 95% CI [0.00, 0.12]; ASIBs: $F_{(1, 214)} = 9.66$, $p = 0.002$, $n_p^2 = 0.04$, 95% CI [0.01, 0.11]). The opposite pattern of responses was seen for LRC infants, decreased (more positive) N290 amplitude in response to faces was related to higher Overall CSS, $F_{(1, 214)} = 20.81$, $p < 0.001$, $n_p^2 = 0.09$, 95% CI [0.03, 0.17]. In response to toys, there was a significant interaction of Overall CSS and group, $F_{(2, 594)} = 9.08$, $p < 0.001$, $n_p^2 = 0.03$, 95% CI [0.01, 0.06]. More negative N290 responses to toys were associated with higher Overall CSS for ASIBs, $F_{(1, 214)} = 28.11$, $p < 0.001$, $n_p^2 = 0.12$, 95% CI [0.05, 0.20]. N290 responses to toys were not significantly associated with Overall CSS for infants with FXS, $p = 0.122$, $n_p^2 = 0.01$, 95% CI [0.00, 0.07], or LRC infants, $p = 0.598$, $n_p^2 = 0.001$, 95% CI [0.00, 0.03].

Figure 2 presents ERP plots, which illustrate the effects described above using a small set of participants at each end of the range of possible scores. Plots labeled "Low Overall CSS" include participants with an Overall CSS of three or less on the ADOS-2. "High Overall CSS" includes participants with an Overall CSS of seven or higher on the ADOS-2. Participants scoring in the mid-range (i.e., CSS of four to six) are not represented in these plots. The N290 is evident as the negative deflection occurring ∼300 ms after stimulus onset. The change in amplitude from the preceding peak of the P1 to the peak of the N290 is greater for ASIBs with high Overall CSS compared with ASIBs with low Overall CSS. N290 amplitude is also greater for participants with FXS and high Overall CSS, particularly in response to faces. LRC participants with low Overall CSS are included for comparison, however, there were too few high-scoring LRC participants for an average ERP plot in the "High Overall CSS" category. In the ERP analyses, ADOS CSS scores were examined continuously. Therefore, these plots do not directly reflect the analyses, but illustrate some of the effects observed in the continuous data.

There was also a significant interaction between participant group, stimulus familiarity, and Overall CSS on N290 amplitude, $F_{(2, 4680)} = 10.26$, $p < 0.001$, $n_p^2 = 0.004$, 95% CI [0.00, 0.01]. We examined responses to familiar and novel stimuli separately. In response to familiar stimuli, there was a significant interaction of Overall CSS and group, $F_{(2,2334)} = 25.43$, $p < 0.001$, $n_p^2 = 0.02$, 95% CI [0.01, 0.03]. As with responses to faces, increased (more negative) N290 amplitude to familiar stimuli were associated with higher Overall CSS for infants with FXS, $F_{(1, 658)} = 9.72$, $p = 0.002$, $n_p^2 = 0.02$, 95% CI [0.00, 0.04], and ASIBs, $F_{(1, 838)} = 24.47$, $p < 0.001$, $n_p^2 = 0.03$, 95% CI [0.01, 0.05], while decreased (more positive) N290 amplitude was associated with higher Overall CSS for LRC infants, $F_{(1, 838)} = 26.07$, $p < 0.001$, $n_p^2 = 0.03$, 95% CI [0.01, 0.06]. There was also a significant interaction of group and Overall CSS in response to novel stimuli, $F_{(2,2346)} = 4.33$, $p = 0.013$, $n_p^2 = 0.004$, 95% CI [0.00, 0.01]. Similar to N290 responses to toys, more negative N290 responses to novel stimuli were associated with higher Overall CSS for ASIBs, $F_{(1, 838)} = 5.79$, $p = 0.0163$, $n_p^2 = 0.01$, 95% CI [0.00, 0.02]. N290 responses to novel stimuli were not associated with Overall CSS for infants with FXS,

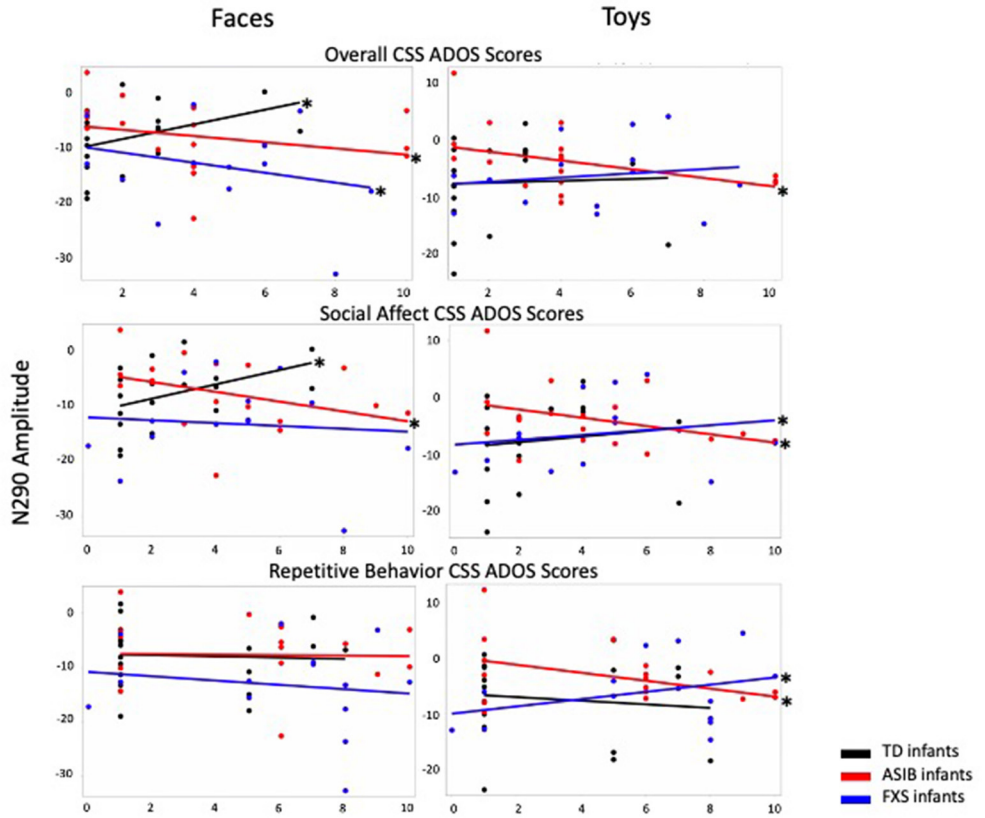

FIGURE 1 | N290 amplitude to faces and toys across ASIBs, infants with FXS, and LRC infants is presented in relation to Overall, Social Affect, and Restricted and Repetitive Behavior calibrated severity scores. Average N290 amplitude is calculated across Parietal Occipital, Lateral Parietal, and Temporal Parietal electrodes.

$p = 0.10$, $n_p^2 = 0.004$, 95% CI [0.00, 0.02], and LRC infants, $p = 0.416$, $n_p^2 = 0.001$, 95% CI [0.00, 0.01].

Social Affect CSS

There was a significant interaction between participant group, stimulus type, and SA CSS on N290 amplitude, $F_{(2, 1188)} = 4.21$, $p = 0.015$, $n_p^2 = 0.01$, 95% CI [0.00, 0.02]. To better understand this interaction, we examined responses to faces and toys separately. There was a significant interaction of SA CSS and group for N290 responses to faces, $F_{(2, 594)} = 16.72$, $p < 0.001$, $n_p^2 = 0.05$, 95% CI [0.02, 0.09]. For ASIBs, a more negative N290 in response to faces was associated with higher SA CSS, $F_{(1, 214)} = 17.53$, $p < 0.001$, $n_p^2 = 0.08$, 95% CI [0.02, 0.15]. Once again, the opposite pattern was seen for LRC infants, who showed that more positive N290 amplitude in response to faces was related to higher SA CSS, $F_{(1, 214)} = 24.49$, $p < 0.001$, $n_p^2 = 0.10$, 95% CI [0.04, 0.18]. For infants with FXS, there was no relationship between N290 responses to faces and SA CSS, $p = 0.384$, $n_p^2 = 0.005$, 95% CI [0.00, 0.05]. There was also a significant interaction of SA CSS and group for N290 responses to toys, $F_{(2, 594)} = 11.35$, $p < 0.001$, $n_p^2 = 0.04$, 95% CI [0.01, 0.07]. More negative N290 responses to toys were associated with higher SA CSS for ASIBs, $F_{(1, 214)} = 20.24$, $p < 0.001$, $n_p^2 = 0.09$, 95% CI [0.03, 0.16], but the opposite pattern was seen for infants with FXS, $F_{(1, 166)} = 4.13$, $p = 0.044$, $n_p^2 = 0.02$, 95% CI [0.00, 0.09]. N290 responses to toys were not significantly associated with SA CSS for LRC infants, $p = 0.088$, $n_p^2 = 0.01$, 95% CI [0.00, 0.06].

There was also a significant interaction between participant group, stimulus familiarity, and SA CSS on N290 amplitude, $F_{(2, 4680)} = 13.45$, $p < 0.001$, $n_p^2 = 0.01$, 95% CI [0.00, 0.01]. We examined responses to familiar and novel stimuli separately. There was a significant interaction of SA CSS and group for N290 responses to familiar stimuli, $F_{(2, 2334)} = 40.03$, $p < 0.001$, $n_p^2 = 0.03$, 95% CI [0.02, 0.05]. A more negative N290 to familiar stimuli was associated with higher SA CSS for infants with FXS, $F_{(1, 658)} = 4.64$, $p = 0.032$, $n_p^2 = 0.01$, 95% CI [0.00, 0.03], and ASIBs, $F_{(1,838)} = 53.96$, $p < 0.001$, $n_p^2 = 0.06$, 95% CI [0.03, 0.09]. More positive N290 amplitude in response to familiar stimuli for LRC infants was related to higher SA CSS, $F_{(1, 838)} = 34.51$, $p < 0.001$, $n_p^2 = 0.04$, 95% CI [0.02, 0.07]. There was a significant interaction of SA CSS and group in response to novel stimuli, $F_{(2, 2346)} = 6.66$, $p = 0.001$, $n_p^2 = 0.01$, 95% CI [0.00, 0.01]. More positive N290 responses to novel stimuli were associated with higher SA CSS for infants with FXS, $F_{(1,658)} = 12.78$, $p < 0.001$, $n_p^2 = 0.02$, 95% CI [0.00, 0.05]. N290 responses to novel stimuli were not associated with SA CSS for ASIBs, $p = 0.225$, $n_p^2 = 0.002$, 95% CI [0.00, 0.01], and LRC infants, $p = 0.065$, $n_p^2 = 0.004$, 95% CI [0.00, 0.02].

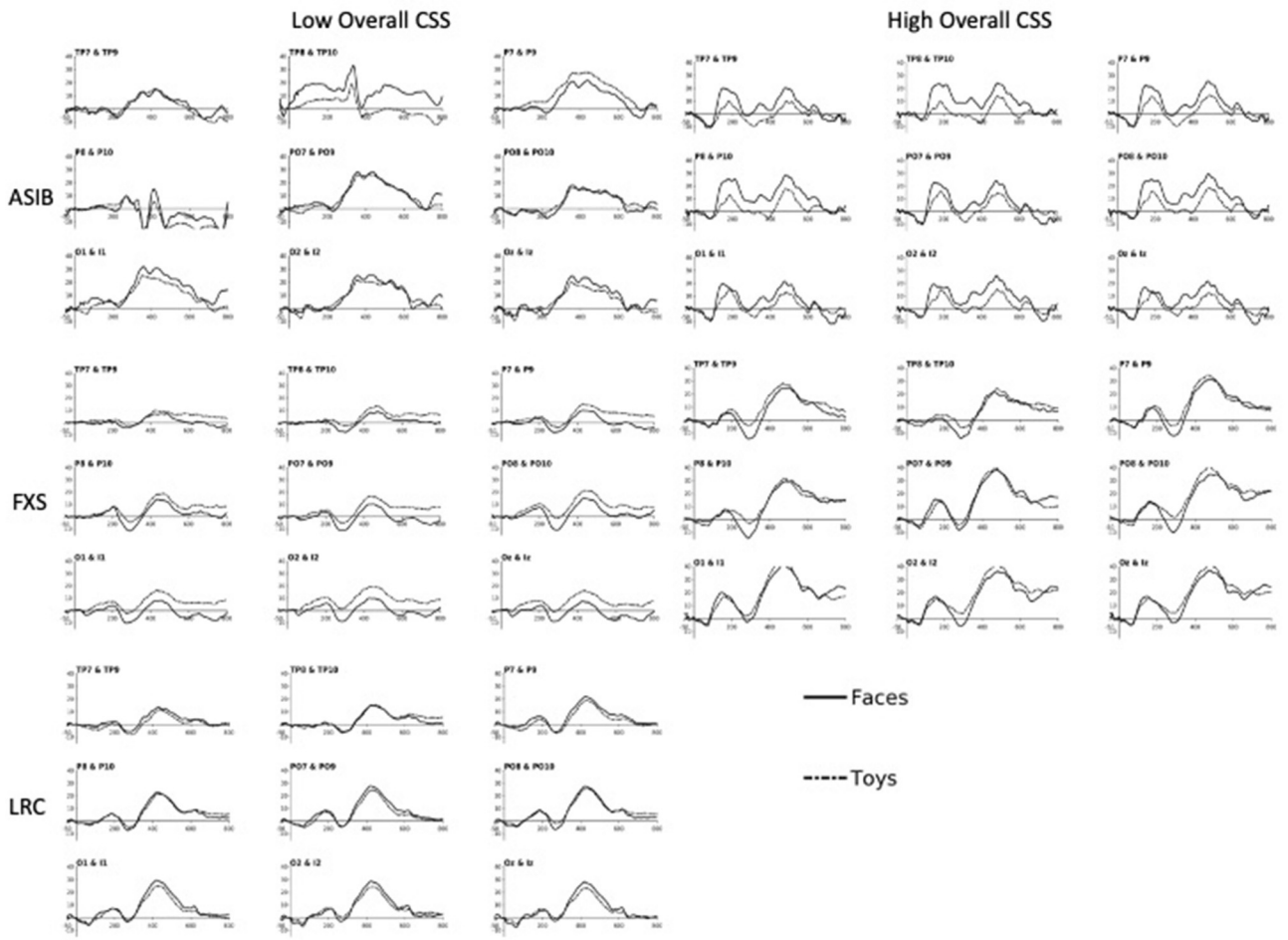

FIGURE 2 | The N290 and P400 responses to faces and toys across subgroups of ASIB, FXS, and LRC participants. Plots labeled "Low Overall CSS" include participants with an Overall CSS of three or less on the ADOS-2. "High Overall CSS" includes participants with an Overall CSS of seven or higher on the ADOS-2. Participants scoring in the mid-range (i.e., scores of four to six) are not represented in these plots. There were not enough LRC participants to create "High Overall CSS" plots. Plots are presented at relevant "virtual 10-10 electrode" clusters, including Temporal Parietal (TP), Parietal (P), Parietal Occipital (PO), Occipital (O), and Inion (I) clusters [see (13, 36) for more details on calculation of 10-10 virtual electrodes].

Restricted and Repetitive Behaviors CSS

There was a significant interaction between participant group, stimulus type, and RRB CSS on N290 amplitude, $F_{(2,\ 1188)} = 9.30$, $p < 0.001$, $n_p^2 = 0.02$, 95% CI [0.00, 0.03]. To better understand this interaction, we examined responses to faces and toys separately. The interaction of RRB CSS and group in response to faces was not significant, $p > 0.05$, $n_p^2 = 0.002$, 95% CI [0.00, 0.01]. However, there was a significant interaction of RRB CSS and group in response to toys, $F_{(2,594)} = 16.38$, $p < 0.001$, $n_p^2 = 0.05$, 95% CI [0.02, 0.09]. More negative N290 responses to toys were associated with higher RRB CSS for ASIBs, $F_{(1,\ 214)} = 28.89$, $p < 0.001$, $n_p^2 = 0.12$, 95% CI [0.05, 0.20], but the opposite pattern was shown by infants with FXS, $F_{(1,166)} = 12.93$, $p < 0.001$, $n_p^2 = 0.07$, 95% CI [0.02, 0.16]. N290 responses to toys were not associated with RRB CSS for LRC infants, $p = 0.144$, $n_p^2 = 0.01$, 95% CI [0.00, 0.05].

There was also a significant interaction between participant group, stimulus familiarity, and RRB CSS on N290 amplitude, $F_{(2,\ 4680)} = 9.27$, $p < 0.001$, $n_p^2 = 0.003$, 95% CI [0.00, 0.01]. We examined responses to familiar and novel stimuli separately. The interaction of RRB CSS and group in response to familiar stimuli was not significant, $F_{(2,\ 2334)} = 1.82$, $p = 0.163$, $n_p^2 = 0.002$, 95% CI [0.00, 0.01]. There was a significant interaction of RRB CSS and group in response to novel stimuli, $F_{(2,\ 2346)} = 14.62$, $p < 0.001$, $n_p^2 = 0.01$, 95% CI [0.01, 0.02]. More negative N290 responses to novel stimuli were associated with higher RRB CSS for ASIBs, $F_{(1,\ 838)} = 13.15$, $p = 0.003$, $n_p^2 = 0.02$, 95% CI [0.00, 0.04], and LRC infants, $F_{(1,850)} = 13.76$, $p < 0.001$, $n_p^2 = 0.02$, 95% CI [0.00, 0.04]. The opposite pattern of responses was seen for infants with FXS, $F_{(1,658)} = 8.85$, $p = 0.003$, $n_p^2 = 0.01$, 95% CI [0.00, 0.04].

P400

Graphs presenting the relations between P400 amplitude to faces and toys across ASIBs, infants with FXS, and LRC infants and Overall, Social Affect, and Restricted and Repetitive Behavior CSS are presented in **Figure 3**.

Overall CSS

There was a significant interaction between participant group, stimulus type, and Overall CSS on P400 amplitude, $F_{(2, 988)} = 4.32$, $p = 0.014$, $n_p^2 = 0.01$, 95% CI [0.00, 0.02]. There was a significant interaction of Overall CSS and group in response to faces, $F_{(2, 494)} = 30.66$, $p < 0.001$, $n_p^2 = 0.11$, 95% CI [0.06, 0.16]. For infants with FXS, a more positive amplitude P400 response to faces was associated with higher Overall CSS, $F_{(1,138)} = 61.39$, $p < 0.001$, $n_p^2 = 0.31$, 95% CI [0.19, 0.42]. However, for LRC infants, decreased (less positive) P400 amplitude in response to faces was related to higher Overall CSS, $F_{(1, 178)} = 4.80$, $p = 0.030$, $n_p^2 = 0.03$, 95% CI [0.00, 0.09]. There was no relation between P400 amplitude in response to faces and Overall CSS for ASIBs, $p = 0.082$, $n_p^2 = 0.02$, 95% CI [0.00, 0.07]. There was a significant interaction of Overall CSS and group in response to toys, $F_{(2, 494)} = 11.86$, $p < 0.001$, $n_p^2 = 0.05$, 95% CI [0.02, 0.08]. For ASIBs decreased (less positive) P400 amplitude in response to toys was related to higher Overall CSS, $F_{(1, 178)} = 9.80$, $p = 0.002$, $n_p^2 = 0.05$, 95% CI [0.01, 0.13]. Once again, for infants with FXS, a more positive P400 in response to toys was associated with higher Overall CSS, $F_{(1, 178)} = 15.74$, $p < 0.001$, $n_p^2 = 0.10$, 95% CI [0.03, 0.20]. There was no relation between P400 amplitude in response to toys and Overall CSS for LRC infants, $p = 0.388$, $n_p^2 = 0.004$, 95% CI [0.00, 0.04]. These effects are observed in the ERP plots presented in **Figure 2**. The P400 is the positive peak occurring at approximately 400–500 ms after stimulus onset. Participants with FXS with high Overall CSS demonstrated a greater amplitude P400 response than those with low Overall CSS. Alternatively, ASIBs with high Overall CSS show a decreased P400 amplitude in response to toys compared to ASIBs with low Overall CSS.

There was also a significant interaction between participant group, stimulus familiarity, and Overall CSS on P400 amplitude, $F_{(2, 3898)} = 14.54$, $p < 0.001$, $n_p^2 = 0.01$, 95% CI [0.00, 0.01]. We examined responses to familiar and novel stimuli separately. There was a significant interaction of Overall CSS and group in response to familiar stimuli, $F_{(2, 1944)} = 8.10$, $p < 0.001$, $n_p^2 = 0.01$, 95% CI [0.00, 0.02]. Infants with FXS showed a more positive P400 associated with higher Overall CSS, $F_{(1, 548)} = 6.41$, $p = 0.012$, $n_p^2 = 0.01$, 95% CI [0.00, 0.04], while ASIBs showed a less positive P400 in response to familiar stimuli was associated with higher Overall CSS, $F_{(1, 698)} = 12.11$, $p < 0.001$, $n_p^2 = 0.02$, 95% CI [0.00, 0.04]. There was no relation between P400 amplitude in response to faces and Overall CSS for LRC infants, $p = 0.790$, $n_p^2 = 0.0001$, 95% CI [0.00, 0.01]. There was a significant interaction of Overall CSS and group in response to novel stimuli, $F_{(2, 1954)} = 48.19$, $p < 0.001$, $n_p^2 = 0.05$, 95% CI [0.03, 0.07]. Infants with FXS showed a more positive P400 associated with higher Overall CSS, $F_{(1, 548)} = 79.40$, $p < 0.001$, $n_p^2 = 0.13$, 95% CI [0.08, 0.18], but ASIBs showed a less positive P400 in response to novel stimuli was associated with higher Overall CSS, $F_{(1, 698)} = 6.34$, $p = 0.012$, $n_p^2 = 0.01$, 95% CI [0.00, 0.03]. There was no relation between P400 amplitude in response to faces and Overall CSS for LRC infants, $p = 0.078$, $n_p^2 = 0.004$, 95% CI [0.00, 0.02].

Social Affect CSS

The interaction between participant group, stimulus type, and SA CSS was not significant, $p = 0.460$, $n_p^2 = 0.002$, 95% CI [0.00, 0.01]. However, there was a significant interaction between participant group and SA CSS on P400 amplitude, $F_{(2, 988)} = 4.00$, $p = 0.019$, $n_p^2 = 0.01$, 95% CI [0.00, 0.02]. For infants with FXS, a higher SA CSS was significantly associated with higher P400 amplitude, $F_{(1, 138)} = 42.09$, $p < 0.001$, $n_p^2 = 0.23$. P400 amplitude was not significantly associated with SA CSS for ASIBs, $p = 0.083$, $n_p^2 = 0.02$, or LRC infants, $p = 0.853$, $n_p^2 = 0.0002$.

There was a significant interaction between participant group, stimulus familiarity, and SA CSS on P400 amplitude, $F_{(2, 3898)} = 14.72$, $p < 0.001$, $n_p^2 = 0.01$, 95% CI [0.00, 0.01]. There was a significant interaction of SA CSS and group in response to familiar stimuli, $F_{(2, 1944)} = 10.28$, $p < 0.001$, $n_p^2 = 0.01$, 95% CI [0.00, 0.02]. Infants with FXS showed a more positive P400 associated with higher SA CSS, F (1, 548) = 5.07, p =0.025, n_p^2 =0.01, 95% CI [.00,0.03], while ASIBs showed a less positive P400 in response to familiar stimuli was associated with higher SA CSS, $F_{(1, 698)} = 13.57$, $p < 0.001$, $n_p^2 = 0.02$, 95% CI [0.00, 0.04]. There was no relation between P400 amplitude in response to familiar stimuli and SA CSS for LRC infants, $p = 0.067$, $n_p^2 = 0.01$, 95% CI [0.00, 0.02]. There was a significant interaction of SA CSS and group in response to novel stimuli, $F_{(2,1954)} = 35.41$, $p < 0.001$, $n_p^2 = 0.04$, 95% CI [0.02, 0.05]. Infants with FXS showed a more positive P400 associated with higher SA CSS, $F_{(1,548)} = 88.91$, $p < 0.001$, $n_p^2 = 0.14$, 95% CI [0.09, 0.19]. There was no relation between P400 amplitude in response to novel stimuli and SA CSS for ASIBs, $p = 0.862$, $n_p^2 = 0.00004$, 95% CI [0.00, 0.01], and LRC infants, $p = 0.401$, $n_p^2 = 0.001$, 95% CI [0.00, 0.01].

Restricted and Repetitive Behavior CSS

The interaction between participant group, stimulus type, and RRB CSS on P400 amplitude was not significant, $F_{(2, 3898)} = 1.45$, $p = 0.235$, $n_p^2 = 0.01$, 95% CI [0.00, 0.02]. However, there was a significant interaction between participant group, stimulus familiarity, and RRB CSS on P400 amplitude, $F_{(2, 3898)} = 33.90$, $p < 0.001$, $n_p^2 = 0.02$, 95% CI [0.01, 0.03]. There was a significant interaction of RRB CSS and group in response to familiar stimuli, $F_{(2, 1944)} = 6.25$, $p = 0.002$, $n_p^2 = 0.01$, 95% CI [0.00, 0.02]. A less positive P400 in response to familiar stimuli was associated with higher RRB CSS for infants with FXS, $F_{(1, 548)} = 27.47$, $p < 0.001$, $n_p^2 = 0.05$, 95% CI [0.02, 0.09], and LRC infants, $F_{(1, 698)} = 15.21$, $p < 0.001$, $n_p^2 = 0.02$, 95% CI [0.01, 0.05]. There was no relation between P400 amplitude in response to familiar stimuli and RRB CSS for ASIBs, $p = 0.200$, $n_p^2 = 0.002$, 95% CI [0.00, 0.02]. There was a significant interaction of RRB CSS and group in response to novel stimuli, $F_{(2, 1954)} = 33.09$, $p < 0.001$, $n_p^2 = 0.03$, 95% CI [0.02, 0.05]. Infants with FXS showed a more positive P400 associated with higher RRB CSS, $F_{(1, 548)} = 18.79$, $p < 0.001$, $n_p^2 = 0.03$, 95% CI [0.01, 0.07]. Less positive P400 amplitudes were associated with higher RRB CSS in ASIBs, $F_{(1, 698)} = 41.16$, $p <$

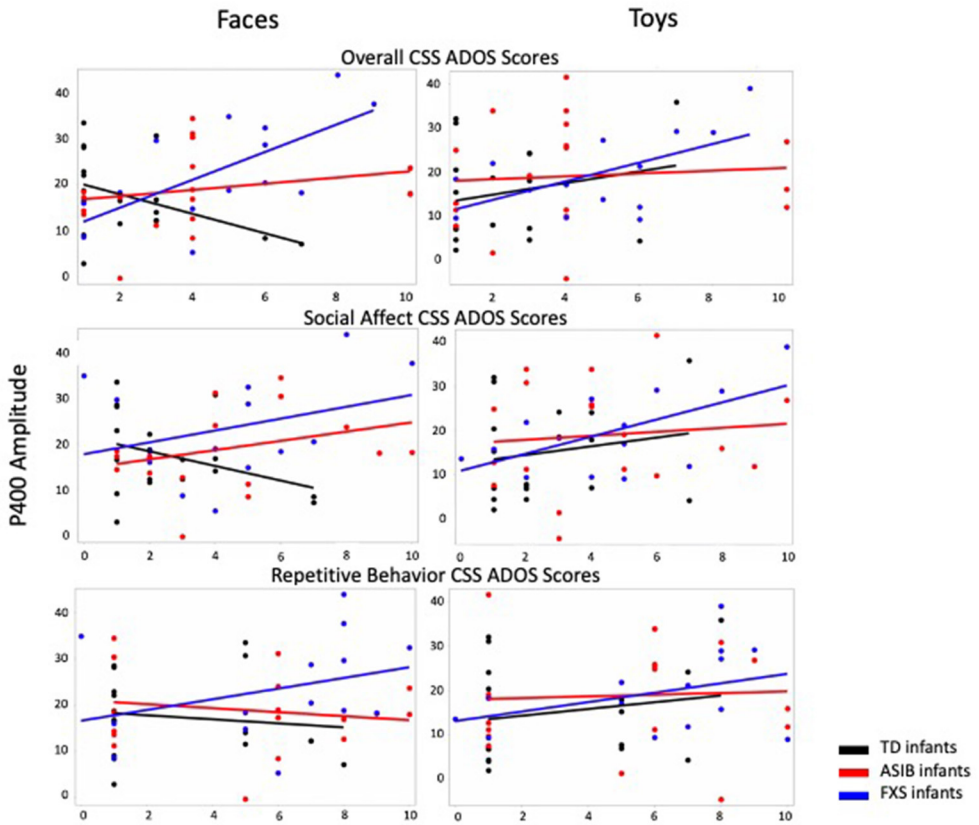

FIGURE 3 | P400 amplitude to faces and toys across ASIBs, infants with FXS, and LRC infants is presented in relation to Overall, Social Affect, and Restricted and Repetitive Behavior calibrated severity scores. Average P400 amplitude is calculated across Parietal Occipital, Occipital, and Inion electrodes.

0.001, $n_p^2 = 0.06$, 95% CI [0.03, 0.09], and LRC infants, $F_{(1, 708)} = 14.93$, $p < 0.001$, $n_p^2 = 0.02$, 95% CI [0.01, 0.05].

Nc

Overall CSS

There were no significant interactions between group, stimulus type, and Overall CSS on Nc amplitude, $F_{(1, 1161)} = 0.09$, $p = 0.914$. There were also no significant interactions between group, stimulus familiarity, and Overall CSS on Nc amplitude, $F_{(1, 1161)} = 0.01$, $p = 0.991$.

Social Affect CSS

There were no significant interactions between group, stimulus type, and SA CSS on Nc amplitude, $F_{(1, 1161)} = 0.02$, $p = 0.979$. Additionally, there were no significant interactions between group, stimulus familiarity, and SA CSS on Nc amplitude, $F_{(1, 1161)} = 1.73$, $p = 0.178$.

Restricted and Repetitive Behavior CSS

There were no significant interactions between group, stimulus type, and RRB CSS on Nc amplitude, $F_{(1, 1161)} = 0.78$, $p = 0.460$. There were also no significant interactions between group, stimulus familiarity, and RRB CSS on Nc amplitude, $F_{(1, 1161)} = 2.48$, $p = 0.085$.

DISCUSSION

Results of the current study establish relations between face processing ERP responses at 12 months of age and ADOS-2 calibrated severity scores in early childhood in FXS as well as siblings of children with ASD and low-risk controls. This work indicates that ERP responses in infancy may be predictive of later behavioral symptoms of ASD in infants at risk for ASD, whether it be syndromic risk or familial risk, and supports the use of ERPs as a measure to identify potential markers of ASD. However, the relations observed were complex and varied across group based on ERP component examined and stimulus category. This likely reflects the high level of heterogeneity in developmental trajectories and symptom profiles associated with ASD, which we were unable to investigate further due to our small sample size.

Results revealed that N290 amplitude was associated with ASD symptoms for all participant groups examined. Infants with FXS showed that greater amplitude (more negative) N290 responses to faces and familiar stimuli were associated with more severe ASD symptoms overall, while less negative N290 responses to toys and novel stimuli were associated with more severe social affect and restricted and repetitive behavior symptoms. ASIBs showed a consistent association between more negative N290 amplitude and greater severity of ASD symptoms. For ASIBs,

more negative N290 amplitude to all stimuli were associated with more severe overall and social affect symptoms, while only more negative N290 amplitude responses to toys and novel stimuli were associated with more severe restricted and repetitive behavior symptoms. The LRC group showed an opposite pattern of responses, such that less negative N290 responses to faces and familiar stimuli were associated with more severe overall and social affect symptoms. The N170 has been implicated as a marker of ASD in children and adults and was recently submitted to the FDA's Center for Drug Evaluation and Research Biomarker Qualification Program with the intent to use N170 responses to better understand heterogeneity in ASD (61). The current work indicates that the N290 may hold promise in distinguishing amongst diverse groups of high-risk infants, as well.

Previous research has indicated that infants with FXS show enhanced N290 ERP activity in response to faces relative to ASIB and LRC groups, while ASIBs showed more muted ERP responses (13). It was hypothesized that these ERP effects may reflect a general hyperactive response to faces in infants with FXS vs. a hypoactive response in ASIBs. Therefore, it was surprising to find that for the both FXS and ASIB participant groups, more negative N290 responses were associated with more severe behavioral symptoms of ASD. This pattern of results was more robust for N290 responses to faces than to toys and to familiar than novel stimuli. When examining relations between N290 responses to toys and novel stimuli and ASD symptom severity, ASIBs continued to show that increased amplitude responses to toys and novel stimuli were associated with more severe symptoms. These results indicate that N290 responses to faces, especially familiar faces, may be informative when investigating ASD risk across discrete etiologies. Furthermore, across all stimulus types, the current results suggest that enhanced responses at the neural level in infancy are more predictive of later developing ASD symptoms than more muted responses.

The P400 amplitude at 12 months of age was also associated with the later development of symptoms of ASD. Greater amplitude P400 responses to all stimuli were associated with more severe overall and social affect symptoms among children with FXS. Decreased P400 amplitude (to familiar stimuli, novel stimuli, and to toys) were associated with higher symptom severity in ASIBs. Typically-developing LRCs showed the opposite effect for P400 responses to faces, where less positive P400 amplitude was linked to more severe ASD symptoms overall. ASIBs showed no significant relations between P400 amplitude to faces and ASD symptom severity. It was surprising that there were strong associations between P400 amplitude and ASD symptom severity in high risk infants, as no significant effects of P400 were observed across participant groups in infancy (13). However, significant effects of participant group and ASD symptom severity on Nc amplitude were not observed.

Relations between the N290 and P400 and later ASD symptoms are consistent with other studies examining links between infant social information processing and ASD outcomes (33). Both the N290 and P400 are believed to reflect infant face processing and enhanced or muted ERP responses may be reflective of atypical social processing. Reduced or atypical patterns of attention to faces have been reported in several behavioral studies of infants later diagnosed with ASD [e.g., (17, 21, 61, 62)]. Reduced engagement during social information processing may disrupt social development, eventually contributing to the development of symptoms of ASD. Gui and colleagues (63) recently reported decreased differentiation of face and object stimuli at the N290 among infants later diagnosed with ASD. Additionally, Shephard and colleagues (64) found that ASIBs with more negative N290 amplitude responses to faces relative to noise scored higher in social communication problems at 7 years of age. Their results indicated that responses to noise stimuli were largely responsible for this effect (i.e., more positive N290 responses were associated with higher social communication problems). Studies by Elsabbagh and colleagues (48) and Buss and colleagues (65) have found that greater P400 amplitude to faces with direct gaze compared with averted gaze was associated with ASD symptoms at 3 years of age. Overall, these findings support our results suggesting that face-sensitive ERPs in infancy may provide insight into later ASD symptoms.

It was surprising that only the face-sensitive N290 and P400 were associated with ASD symptoms, and not the Nc ERP component. The Nc was differentiated across all three groups in infancy (13), and we had expected that unqiue patterns of Nc responses may be associated with unique symptom profiles. The Nc is associated with engagement of attention, and differences in Nc amplitude have been reported between infants at high risk for ASD and control participants during infancy [e.g., (28, 30, 65, 66)]. Although Nc amplitude has been associated with infant risk status, its relation to ASD outcomes is less clear. Our results are consistent with one recent study finding no connection between mean Nc amplitude at 8 months of age and later ASD (67). Another study reported differences in Nc amplitude between 6-month-old infants later diagnosed with ASD and control participants, but not 12-month-olds later diagnosed with ASD and control participants (68). We had expected that Nc amplitude would be associated with ASD symptoms in our sample of FXS, ASIBs, and LRC infants, due to unique patterns of Nc activation observed across these groups (13), but all of the current Nc analyses were nonsignificant. Relations between Nc amplitude and ASD outcomes should be further investigated in future research, due to sparse and conflicting results.

Additional work in this area is needed to better understand how neural responses differ across infants with FXS based on ASD diagnosis and how infants with FXS that receive an ASD diagnosis uniquely process stimuli from ASIBs that receive an ASD diagnosis. Small sample size was a significant limitation in the current study. While current sample sizes restricted us from analyzing outcomes categorically, we aim to expand our sample to better understand how infant ERP responses across these groups are associated with diagnostic outcomes in future research. Further work in this area with larger samples will allow us to examine the role of high-risk subgroups (e.g., sex, cognitive function) in infant ERP responses and ASD outcomes. There were more females (i.e., 50%) in the sample of children with FXS in the current study than is typical. This may have impacted our results, as a smaller subset was diagnosed was diagnosed with

ASD than has been reported in larger scale studies of ASD in preschool children with FXS (i.e., 36 vs. 61%; 9). Additionally, the inclusion of multiple comparisons in our statistical approach may have allowed for Type I errors. Methods were adopted to help to prevent Type I errors and effect sizes, including 95% confidence intervals for effect sizes, were reported to further describe comparisons, however, replication of this work will be important to confirmation of the current findings. Additional work with a large sample of infants with FXS would allow for investigation of the role of gender in relations between infant ERP responses and later developing symptoms of ASD. Furthermore, FXS is associated with intellectual impairment, and it is important to examine how the presence of intellectual disability may impact stimulus processing in this sample. Adoption of additional control groups that are impacted by intellectual disability, but less likely to develop ASD, such as infants with Down syndrome, may allow for better understanding of how unique patterns of neural responses in infants with FXS are associated with intellectual disability. Another goal of this line of research is to expand the investigation to other high-risk participant groups. For example, Feldman and colleagues (69) found that infants in families with a greater number of medical conditions were more likely to demonstrate ASD symptoms. It would be valuable to investigate neural responses and ASD symptoms in this and other high-risk groups to better understand heterogeneous pathways to ASD.

Results of the current study highlight that neural correlates of face processing in infancy are associated with later behavioral symptoms of ASD in children with FXS. This work is highly valuable, especially as this was the first study to examine relations between infant ERPs and symptoms of ASD in a high-risk group beyond ASIBs. By including multiple groups of participants at high risk for ASD, we were able to examine heterogeneity in relations between infant neural responses and childhood ASD symptoms. Despite the observation of unique patterns of neural responses in infancy across infants with FXS and ASIBs, similar patterns of infant ERP responses were associated with childhood ASD symptom severity for both groups. Specifically, we found that greater N290 amplitude in response to faces in infancy was associated with the presentation of more severe symptoms of ASD in early childhood across both infants with FXS and ASIBs. However, less consistent results were observed across high-risk groups for the P400 and Nc components. It will be important to further investigate the utility of the N290, P400, and Nc ERP components as potential markers for ASD in future studies enrolling large and diverse high-risk samples. It is our intention that this work will eventually contribute to the ability to identify valid and reliable ERP markers evident at the level of the individual, promoting early intervention and treatment among infants and toddlers most likely to receive a diagnosis of ASD.

DATA AVAILABILITY STATEMENT

The clinical and demographic data for this study are available as part of the National Database for Autism Research (https://nda.nih.gov/edit_collection.html?id=1888). Contact the authors to access additional data.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of South Carolina Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

MG, JRi, AH, and JRo contributed to the conception and design of the study. JRi and JRo led recruitment and data collection efforts. AH organized the clinical data and assisted in its use and interpretation. MG performed the statistical analyses, created the figures and table, and wrote the first draft of the manuscript. All authors contributed to manuscript revision and approved the submitted version.

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REFERENCES

1. Coffee B, Keith K, Albizua I, Malone T, Mowrey J, Sherman SL, et al. Incidence of fragile X syndrome by newborn screening for methylated FMR1 DNA. *Am J Hum Genet.* (2009) 85:503–14. doi: 10.1016/j.ajhg.2009.09.007
2. Crawford DC, Acuña JM, Sherman SL. FMR1 and the fragile X syndrome: human genome epidemiology review. *Genet Med.* (2001) 3:359–71. doi: 10.1097/00125817-200109000-00006
3. Hatton DD, Sideris J, Skinner M, Mankowski J, Bailey DB, Roberts J, et al. Autistic behavior in children with fragile X syndrome: prevalence, stability, and the impact of FMRP. *Am J Med Genet A.* (2006) 140A:1804–13. doi: 10.1002/ajmg.a.31286
4. Hunter J, Rivero-Arias O, Angelov A, Kim E, Fotheringham I, Leal J. Epidemiology of fragile X syndrome: a systematic review and meta-analysis. *Am J Med Genet A.* (2014) 164:1648–58. doi: 10.1002/ajmg.a.36511
5. Clifford S, Dissanayake C, Bui QM, Huggins R, Taylor AK, Loesch DZ. Autism spectrum phenotype in males and females with fragile X full mutation and premutation. *J Autism Dev Disord.* (2007) 37:738–47. doi: 10.1007/s10803-006-0205-z
6. Harris SW, Hessl D, Goodlin-Jones B, Ferranti J, Bacalman S, Barbato I, et al. Autism profiles of males with fragile X syndrome. *Am J Ment Retard.* (2008) 113:427–38. doi: 10.1352/2008.113:427-438
7. Kaufmann WE, Cortell R, Kau ASM, Bukelis I, Tierney E, Gray RM, et al. Autism spectrum disorder in fragile X syndrome: communication, social interaction, and specific behaviors. *Am J Med Genet.* (2004) 129A:225–34. doi: 10.1002/ajmg.a.30229
8. Philofsky A, Hepburn SL, Hayes A, Hagerman R, Rogers SJ. Linguistic and cognitive functioning and autism symptoms in young children with fragile X syndrome. *Am J Ment Retard.* (2004) 109:208–18. doi: 10.1352/0895-8017(2004)109<208:LACFAA>2.0.CO;2
9. Roberts JE, Bradshaw J, Will E, Hogan AL, McQuillin S, Hills K. Emergence and rate of autism in fragile X syndrome across the first years of life. *Dev Psychopathol.* (2020) 32:1335–52. doi: 10.1017/S0954579420000942
10. Shaw KA, Maenner MJ, Baio J, Washington A, Christensen DL, Wiggins LD, et al. Early identification of autism spectrum disorder among children

aged 4 Years-early autism and developmental disabilities monitoring network, six sites, United States, (2016). *MMWR Surveill Summ.* (2020) 69:1–11. doi: 10.15585/mmwr.ss6903a1
11. Hogan AL, Caravella KE, Ezell J, Rague L, Hills K, Roberts JE. Autism spectrum disorder symptoms in infants with fragile X syndrome: a prospective case series. *J Autism Dev Disord.* (2017) 47:1628–44. doi: 10.1007/s10803-017-3081-9
12. Roberts JE, Tonnsen BL, McCary LM, Caravella KE, Shinkareva SV. Brief Report: Autism Symptoms in Infants with Fragile X Syndrome. *J Autism Dev Disord*. (2016) 46:3830–7. doi: 10.1007/s10803-016-2903-5
13. Guy MW, Richards JE, Tonnsen BL, Roberts JE. Neural correlates of face processing in etiologically-distinct 12-month-old infants at high-risk of autism spectrum disorder. *Dev Cogn Neurosci.* (2018) 29:61–71. doi: 10.1016/j.dcn.2017.03.002
14. Messinger D, Young GS, Ozonoff S, Dobkins K, Carter A, Zwaigenbaum L, et al. Beyond autism: a baby siblings research consortium study of high-risk children at three years of age. *J Am Acad Child Adolesc Psychiatry.* (2013) 52:300-308.e1. doi: 10.1016/j.jaac.2012.12.011
15. Ozonoff S, Young GS, Carter A, Messinger D, Yirmiya N, Zwaigenbaum L, et al. Recurrence risk for autism spectrum disorders: a baby siblings research consortium study. *Pediatrics.* (2011) 128:e488–95. doi: 10.1542/peds.2010-2825
16. Szatmari P, Chawarska K, Dawson G, Georgiades S, Landa R, Lord C, et al. Prospective longitudinal studies of infant siblings of children with autism: lessons learned and future directions. *J Am Acad Child Adolesc Psychiatry.* (2016) 55:179–87. doi: 10.1016/j.jaac.2015.12.014
17. Chawarska K, Macari S, Shic F. Decreased spontaneous attention to social scenes in 6-month-old infants later diagnosed with autism spectrum disorders. *Biol Psychiatry.* (2013) 74:195–203. doi: 10.1016/j.biopsych.2012.11.022
18. Shic F, Macari S, Chawarska K. Speech disturbs face scanning in 6-month-old infants who develop autism spectrum disorder. *Biol Psychiatry.* (2014) 75:231–7. doi: 10.1016/j.biopsych.2013.07.009
19. Di Giorgio E, Frasnelli E, Rosa Salva O, Maria Luisa S, Puopolo M, Tosoni D, et al. Difference in visual social predispositions between newborns at low- and high-risk for autism. *Sci Rep.* (2016) 6:26395. doi: 10.1038/srep26395
20. Elsabbagh M, Gliga T, Pickles A, Hudry K, Charman T, Johnson MH. The development of face orienting mechanisms in infants at-risk for autism. Behav Brain Res. (2013) 251:147–54. doi: 10.1016/j.bbr.2012.07.030
21. Elsabbagh M, Bedford R, Senju A, Charman T, Pickles A, Johnson MH, et al. What you see is what you get: contextual modulation of face scanning in typical and atypical development. *Soc Cogn Affect Neurosci.* (2014) 9:538–43. doi: 10.1093/scan/nst012
22. Roberts JE, Crawford H, Will EA, Hogan AL, McQuillin S, Tonnsen BL, et al. Infant social avoidance predicts autism but not anxiety in fragile X syndrome. *Front Psychiatry.* (2019) 10:199. doi: 10.3389/fpsyt.2019.00199
23. Roberts JE, Hatton DD, Long ACJ, Anello V, Colombo J. Visual attention and autistic behavior in infants with fragile X syndrome. *J Autism Dev Disord.* (2012) 42:937–46. doi: 10.1007/s10803-011-1316-8
24. Roberts JE, Tonnsen B, Robinson A, Shinkareva SV. Heart activity and autistic behavior in infants and toddlers with fragile X syndrome. *Am J Intellect Dev Disabil.* (2012) 117:90–102. doi: 10.1352/1944-7558-117.2.90
25. Mccary LM, Roberts JE. Early identification of autism in fragile X syndrome: a review. *J Intellect Disabil Res.* (2013) 57:803–14. doi: 10.1111/j.1365-2788.2012.01609.x
26. Bosl WJ, Tager-Flusberg H, Nelson CA. EEG analytics for early detection of autism spectrum disorder: a data-driven approach. *Sci Rep.* (2018) 8. doi: 10.1038/s41598-018-24318-x
27. di Lorenzo R, Munsters NM, Ward EK, de Jonge M, Kemner C, van den Boomen C. Is it fear? similar brain responses to fearful and neutral faces in infants with a heightened likelihood for autism spectrum disorder. *J Autism Dev Disord.* (2021) 51:961–72. doi: 10.1007/s10803-020-04560-x
28. Key APF, Stone WL. Same but different: 9-month-old infants at average and high risk for autism look at the same facial features but process them using different brain mechanisms. *Autism Res.* (2012) 5:253–66. doi: 10.1002/aur.1231
29. Luyster RJ, Powell C, Tager-Flusberg H, Nelson CA. Neural measures of social attention across the first years of life: characterizing typical development and markers of autism risk. *Dev Cogn Neurosci.* (2014) 8:131–43. doi: 10.1016/j.dcn.2013.09.006
30. McCleery JP, Akshoomoff N, Dobkins KR, Carver LJ. Atypical face versus object processing and hemispheric asymmetries in 10-month-old infants at risk for autism. *Biol Psychiatry.* (2009) 66:950–7. doi: 10.1016/j.biopsych.2009.07.031
31. Johnson MH, Griffin R, Csibra G, Halit H, Farroni T, de Haan M, et al. The emergence of the social brain network: evidence from typical and atypical development. *Dev Psychopathol.* (2005) 17:599–619. doi: 10.1017/S0954579405050297
32. Johnson MH, Senju A, Tomalski P. The two-process theory of face processing: modifications based on two decades of data from infants and adults. *Neurosci Biobehav Rev.* (2015) 50:169–79. doi: 10.1016/j.neubiorev.2014.10.009
33. Klin A, Shultz S, Jones W. Social visual engagement in infants and toddlers with autism: Early developmental transitions and a model of pathogenesis. *Neurosci Biobehav Rev.* (2015) 50:189–203. doi: 10.1016/j.neubiorev.2014.10.006
34. Ozonoff S, Iosif AM. Changing conceptualizations of regression: What prospective studies reveal about the onset of autism spectrum disorder. *Neurosci Biobehav Rev.* (2019) 100:296–304. doi: 10.1016/j.neubiorev.2019.03.012
35. Conte S, Richards JE, Guy MW, Xie W, Roberts JE. Face-sensitive brain responses in the first year of life. *NeuroImage.* (2020) 211:116602. doi: 10.1016/j.neuroimage.2020.116602
36. Guy MW, Zieber N, Richards JE. The cortical development of specialized face processing in infancy. *Child Dev.* (2016) 87:1581–600. doi: 10.1111/cdev.12543
37. Halit H, Csibra G, Volein Á, Johnson MH. Face-sensitive cortical processing in early infancy. *J Child Psychol Psychiatry.* (2004) 45:1228–34. doi: 10.1111/j.1469-7610.2004.00321.x
38. de Haan M, Johnson MH, Halit H. Development of face-sensitive event-related potentials during infancy: A review. *Int J Psychophysiol.* (2003) 51:45–58. doi: 10.1016/S0167-8760(03)00152-1
39. de Haan M, Nelson CA. Brain activity differentiates face and object processing in 6-month-old infants. *Dev Psychol.* (1999) 35:1113–21. doi: 10.1037/0012-1649.35.4.1113
40. Courchesne E, Ganz L, Norcia AM. Event-related brain potentials to human faces in infants. *Child Dev.* (1981) 52:804–11. doi: 10.2307/1129080
41. de Haan M, Nelson CA. Recognition of the mother's face by six-month-old infants: a neurobehavioral study. *Child Dev.* (1997) 68:187–210. doi: 10.1111/j.1467-8624.1997.tb01935.x
42. Guy MW, Reynolds GD, Zhang D. Visual attention to global and local stimulus properties in 6-month-old infants: individual differences and event-related potentials. *Child Dev.* (2013) 84:1392–406. doi: 10.1111/cdev.12053
43. Webb SJ, Long JD, Nelson CA. A longitudinal investigation of visual event-related potentials in the first year of life. *Dev Sci.* (2005) 8:605–16. doi: 10.1111/j.1467-7687.2005.00452.x
44. Reynolds GD, Richards JE. Familiarization, attention, and recognition memory in infancy: An event-related potential and cortical source localization study. *Dev Psychol.* (2005) 41:598–615. doi: 10.1037/0012-1649.41.4.598
45. Reynolds GD, Courage ML, Richards JE. Infant attention and visual preferences: converging evidence from behavior, event-related potentials, and cortical source localization. *Dev Psychol.* (2010) 46:886–904. doi: 10.1037/a0019670
46. Cordeiro L, Ballinger E, Hagerman R, Hessl D. Clinical assessment of DSM-IV anxiety disorders in fragile X syndrome: prevalence and characterization. *J Neurodev Disord.* (2011) 3:57–67. doi: 10.1007/s11689-010-9067-y
47. Clements CC, Zoltowski AR, Yankowitz LD, Yerys BE, Schultz RT, Herrington JD. Evaluation of the social motivation hypothesis of autism a systematic review and meta-analysis. *JAMA Psychiatry*. (2018) 75:797–808. doi: 10.1001/jamapsychiatry.2018.1100
48. Elsabbagh M, Holmboe K, Gliga T, Mercure E, Hudry K, Charman T, et al. Social and attention factors during infancy and the later emergence of autism characteristics. In: Braddick O, Atkinson J, Innocenti GM, editors.

Gene Expression to Neurobiology and Behaviour: Human Brain Development and Developmental Disorders Amsterdam: Elsevier (2012). p. 195–207.
49. Charman T, Taylor E, Drew A, Cockerill H, Brown JA, Baird G. Outcome at 7 years of children diagnosed with autism at age 2: predictive validity of assessments conducted at 2 and 3 years of age and pattern of symptom change over time. *J Child Psychol Psychiatry.* (2005) 46:500–13. doi: 10.1111/j.1469-7610.2004.00377.x
50. Turner LM, Stone WL. Variability in outcome for children with an ASD diagnosis at age 2. *J Child Psychol Psychiatry.* (2007) 48:793–802. doi: 10.1111/j.1469-7610.2007.01744.x
51. Guthrie W, Swineford LB, Nottke C, Wetherby AM. Early diagnosis of autism spectrum disorder: stability and change in clinical diagnosis and symptom presentation. *J Child Psychol Psychiatry.* (2013) 54:582–90. doi: 10.1111/jcpp.12008
52. Ozonoff S, Young GS, Landa RJ, Brian J, Bryson S, Charman T, et al. Diagnostic stability in young children at risk for autism spectrum disorder: a baby siblings research consortium study. *J Child Psychol Psychiatry.* (2015) 56:988–98. doi: 10.1111/jcpp.12421
53. Pierce K, Gazestani VH, Bacon E, Barnes CC, Cha D, Nalabolu S, et al. Evaluation of the diagnostic stability of the early autism spectrum disorder phenotype in the general population starting at 12 months. *JAMA Pediatrics.* (2019) 173:578–87. doi: 10.1001/jamapediatrics.2019.0624
54. Lord C, Rutter M, DiLavore P, Risi S, Gotham K, Bishop S. *Autism Diagnostic Observation Schedule, Second Edition: ADOS-2.* Torrance, CA: Western Psychological Services (2012).
55. Gotham K, Pickles A, Lord C. Standardizing ADOS scores for a measure of severity in autism spectrum disorders. *J Autism Dev Disord.* (2009) 39:693–705. doi: 10.1007/s10803-008-0674-3
56. Hus V, Gotham K, Lord C. Standardizing ADOS domain scores: separating severity of social affect and restricted and repetitive behaviors. *J Autism Dev Disord.* (2014) 44:2400–12. doi: 10.1007/s10803-012-1719-1
57. Shumway S, Farmer C, Thurm A, Joseph L, Black D, Golden C. The ADOS calibrated severity score: Relationship to phenotypic variables and stability over time. *Autism Res.* (2012) 5:267–76. doi: 10.1002/aur.1238
58. Delorme A, Makeig S. EEGLAB: an open source toolbox for analysis of single-trial EEG dynamics including independent component analysis. *J Neurosci Methods.* (2004) 134:9–21. doi: 10.1016/j.jneumeth.2003.10.009
59. Lopez-Calderon J, Luck SJ. ERPLAB: an open-source toolbox for the analysis of event-related potentials. *Front Hum Neurosci.* (2014) 8:213. doi: 10.3389/fnhum.2014.00213
60. Guy MW, Conte S, Bursalioglu A, Richards JE. Peak selection and latency jitter correction in developmental event-related potentials. *Dev Psychol.* (2021) 63:e22193. doi: 10.1002/dev.22193
61. McPartland JC, Bernier RA, Jeste SS, Dawson G, Nelson CA, Chawarska K, et al. The autism biomarkers consortium for clinical trials (ABC-CT): scientific context, study design, and progress toward biomarker qualification. *Front Integr Neurosci.* (2020) 14:16. doi: 10.3389/fnint.2020.00016
62. Jones W, Klin A. Attention to eyes is present but in decline in 2–6-month-old infants later diagnosed with autism. *Nature.* (2013) 504:427–31. doi: 10.1038/nature12715
63. Gui A, Meaburn EL, Tye C, Charman T, Johnson MH, Jones EJH. Association of polygenic liability for autism with face-sensitive cortical responses from infancy. *JAMA Pediatr.* (2021) 175:968–70. doi: 10.1001/jamapediatrics.2021.1338
64. Shephard E, Milosavljevic B, Mason L, Elsabbagh M, Tye C, Gliga T, et al. Neural and behavioural indices of face processing in siblings of children with autism spectrum disorder (ASD): A longitudinal study from infancy to mid-childhood. *Cortex.* (2020) 127:162–79. doi: 10.1016/j.cortex.2020.02.008
65. Bussu G, Llera A, Jones EJH, Tye C, Charman T, Johnson MH, et al. Uncovering neurodevelopmental paths to autism spectrum disorder through an integrated analysis of developmental measures and neural sensitivity to faces. J Psychiatry Neurosci. (2021) 46:E34–43. doi: 10.1503/jpn.190148
66. Key AP, Ibanez LV, Henderson HA, Warren Z, Messinger DS, Stone WL. Positive affect processing and joint attention in infants at high risk for autism: an exploratory study. *J Autism Dev Disord.* (2015) 45:4051–62. doi: 10.1007/s10803-014-2191-x
67. Gui A, Bussu G, Tye C, Elsabbagh M, Pasco G, Charman T, et al. Attentive brain states in infants with and without later autism. *Transl Psychiatry.* (2021) 11:196. doi: 10.1038/s41398-021-01368-w
68. Jones EJH, Venema K, Earl R, Lowy R, Barnes K, Estes A, et al. Reduced engagement with social stimuli in 6-month-old infants with later autism spectrum disorder: a longitudinal prospective study of infants at high familial risk. *J Neurodev Disord.* (2016) 8. doi: 10.1186/s11689-016-9139-8
69. Feldman MA, Azzano A, Ward RA, Hudson M, Sjaarda CP, Liu X. Relationship of family history conditions and early signs of autism spectrum disorder in low and high-risk infants. *Res Autism Spectr Disord.* (2019) 65:25–33. doi: 10.1016/j.rasd.2019.05.002

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MINI REVIEW
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How Knowledge Mapping Can Bridge the Communication Gap Between Caregivers and Health Professionals Supporting Individuals With Complex Medical Needs: A Study in Fragile X Syndrome

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The challenges of caring for children with complex health needs, such as intellectual disability (ID) and autism spectrum disorder (ASD), are multiple and experienced by both caregivers and health professionals. Fragile X syndrome (FXS) is the most common single gene cause of ID and ASD, and provides a pertinent model to understand these complexities of care, as well as the communication challenges experienced between caregivers and healthcare professionals. In recent years both caregivers and healthcare professionals have recognized the need for enhancing communication both in clinical and research settings. Knowledge mapping has emerged as a tool to support quality communication between team participants. Here we review how differences in mental models, as well as challenges related to health literacy and knowledge transfer can have an impact on communication. Next, we present different knowledge mapping approaches used in complex situations, with a focus on concept maps and care maps. Finally, we highlight the potential benefits and limitations of mapping to improve communication issues related to caring for individuals with FXS and potentially other neurodevelopmental disorders (NDDs).

Keywords: Fragile X syndrome, care mapping, communication, mental model, medical complexity, knowledge mapping, concept mapping

INTRODUCTION

Neurodevelopmental Disorders Represent a Prevalent Case of Medical Complexity

Among the many conditions associated with pediatric medical complexity, neurodevelopmental disability (NDD) is one of the most notable as they affect 3–18% of the world's population (1–7). NDDs include conditions such as Attention Deficit/Hyperactivity disorder (ADHD), learning disability, intellectual disability (ID), and autism spectrum disorder (ASD). Most individuals with an

NDD not only have multiple core symptoms linked to their condition but also experience related (comorbid) conditions, and as such are usually followed by a large team of health and education specialists (8). Having multiple partners involved in care coordination can create challenges in communication. Quality communication in healthcare is essential to ensure a high standard of care; poor communication often results in unmet family needs, dissatisfaction in care, and potentially even medical errors or needless interventions (9–11).

The complexity in needs and services for individuals with NDDs tasks caregivers and healthcare professionals with enormous responsibilities (12). For caregivers, the impact of managing their child's multiple health complexities, the lack of training to provide expert care, and grief can contribute to an overall feeling of helplessness and exhaustion (13, 14). Additionally, caregivers encounter financial challenges and can struggle with depression (15–17). Caregivers often become a "manager" for their child: advocating for and coordinating their child's care, managing communication between healthcare and social providers, implementing therapeutic recommendations, and maintaining their child's medical health information and records (16–18). For healthcare professionals, children living with multiple health complexities belong to a unique population that demands ongoing continuous care and utilizes a disproportionate amount of healthcare services (12, 13, 16, 17, 19–22). In addition, healthcare professionals can be required to provide care that often extends beyond their capacity due to limitations in skills, psychosocial support, opportunities for continuing education, or lack of resources (13, 23, 24).

Fragile X Syndrome as a Model for Complex Care in NDD

In this review we focus on Fragile X syndrome (FXS) as it is the most common single gene cause of ASD and ID. More importantly, FXS illustrates the complexity of symptoms and the challenges in communication between caregivers and healthcare professionals seen in most NDDs. Indeed, individuals with FXS present clinically with a wide spectrum of symptoms and comorbid conditions, including core cognitive and adaptive function challenges (25), speech delay (26, 27), autism spectrum traits (28–31), sleep issues (32–34), challenging behavior (35), anxiety (36), and mental health issues (37). FXS is also an excellent model for understanding changing needs over a lifespan as, like most NDDs, it is a lifelong condition where the health, educational, and social needs of individuals evolve over time, making care even more complex and challenging for parents and health professionals (38). As a child diagnosed with FXS enters each life stage, new symptoms and behaviors can appear while existing ones can potentially intensify (39). As the child transitions to adult services, parents take on the responsibility of initiating and transferring services and funding from youth to adult programs (38, 40–44). There is no gold-standard model for transition from pediatric care to adult care, making the transition to adult services a struggle for families to know what to do. Limited access to services can create anxiety and decreased quality of life for both parents and individuals living with FXS (38, 45, 46).

Finally, FXS highlights the importance of considering the family unit when assessing complexity in care and communication. In FXS, mothers can have a range of genetic variants from carriers of a pre-mutation which does not manifest as FXS but can lead to biological (e.g., early menopause) and psychological (e.g., anxiety and depression) challenges to full mutation with FXS symptoms, which need to be considered during the communication process.

Through a review of the literature, this mini-review summarizes the key components impacting communication between caregivers and healthcare professionals, and explores the use of knowledge mapping as a tool to strengthen quality communication. Our goal is to evaluate these challenges in communication through the lens of care needed to support individuals with FXS. We will discuss: (1) the key components impacting communication between caregivers and healthcare providers in FXS and other medically complex situations, (2) ways to visually represent and share complex information, known as knowledge mapping, as a method to enhance communication, and (3) how mapping has been shown to improve communication in complex situations.

Our central hypothesis is that communication challenges between caregivers and health professionals may be improved with the implementation of knowledge mapping.

METHODS

In order to better understand how to improve the quality of communication between caregivers and healthcare professionals, we performed a 2-step literature review using a pragmatic approach. The most relevant papers are cited. First, we conducted a general literature review in order to identify the components impacting communication between caregivers and healthcare professionals in the context of children with complex needs. We used the following keywords to search English language text from PubMed and Google Scholar with no limitation on time period: "*healthcare professionals, physicians, communication, caregivers, parents, patients, pediatric healthcare, complex medical needs, neurodevelopmental disabilities, NDD, intellectual disability, ID, Fragile X syndrome, FXS, attention deficit/hyperactivity disorder, ADHD, autism spectrum disorder, and ASD.*" We included both original articles and reviews, and identified 68 papers. We reviewed those papers and identified converging themes centered around "*mental model, health literacy, knowledge transfer, beliefs, perspectives, mapping, concept mapping, and care mapping.*"

Next, we focused on mapping methods for concepts relevant to complex situations and healthcare, searching for articles discussing "*communication, mapping, knowledge mapping, mental models, concept mapping, and care mapping.*" We identified 46 papers and 3 books which were used to prepare the themes of review.

RESULTS

Components Impacting Communication Between Caregivers and Healthcare Professionals in FXS and Other Medically Complex Situations

While there are many factors leading to communication challenges in complex medical situations, we found that a key aspect resided in the concepts and categories individuals have developed over time. Challenges in communication present themselves when individuals have conflicting concepts and categories. This is known as lack of coherence in mental models. Two factors contributing to the difference in mental models were the base knowledge of individuals, also known as literacy, and their ability to exchange information, referred to as knowledge transfer. We discuss below those 3 interlinked concepts.

Coherence in Mental Models of Caregivers and Healthcare Professionals Drive Quality Communication

A person's understanding of concepts and their relation to other concepts is formed by past experiences, education, and perceived knowledge, and is referred to as a mental model (47). Mental models are also influenced by multiple factors including family status, cultural beliefs, education, literacy, and goals (48, 49). Mental models play a significant role in an individual's decision making and behavior, as well as communication. In **Figure 1** we use a concept map as a tool to visually represent a caregiver's mental model (**1A**) and a healthcare professional's mental model (**1B**) to show how mental models can vary between caregivers and healthcare professionals supporting individuals living with FXS.

In healthcare, research in obesity, nursing, clinical teamwork, and oncology has identified how understanding individual mental models has the ability to increase efficiency in team performance when mental models are shared (48, 50–53). It also showed that shared understanding of an individual's mental model prevents communication errors and opens the door for effective communication, collaboration, and navigation within the health system (50, 52). Researchers have also studied the mental models of patients with varying mental health conditions such as depression, obsessive compulsive disorder, bipolar disorder, and schizophrenia to better understand the condition but also clinical care (54–59).

Mental models are variable between individuals, an important issue to acknowledge for optimal communication. Surveys of caregivers supporting individuals with FXS showed an important diversity in perceptions of the impact of a FXS diagnosis, burden of caring for a child with FXS, and decision making (60–63). Professionals working in healthcare present with variability in mental models too. When comparing the mental models of obesity between healthcare professionals and policymakers, Sturgiss et al. identified a clear distinction between each group (48).

Health Literacy Contributes Significantly to Mental Model

A key building block of a mental model is health literacy, which is defined as "the ability to access, understand, evaluate, and communicate information in a way to promote, maintain and improve health in a variety of settings across the life-course" (44). While limitations in space do not allow for a full review of the large number of studies in health literacy (64, 65), we highlight below how literacy impacts communication. Indeed, a caregiver's health literacy has a direct impact on the health of their children (66, 67). Caregivers with low health literacy lack the knowledge to associate appropriate health services with specific care needs, and experience a disconnect in communication and flow of information with healthcare professionals (13). Interestingly, a caregiver's health literacy has the potential to evolve with time through trial and error, extensive research, online resources, peer recommendations, and analysis of journal articles (68, 69), which can in turn lead to evolution of the mental model of that individual. In FXS, as for many other medical conditions, another aspect to consider is that a large amount of information must be gained rapidly. For instance, a caregiver whose child is diagnosed with FXS will need to learn about FXS but also linked diagnosis related to carrier status (70).

Additionally, when a caregiver assumes their healthcare provider has a lack of health literacy around their child's diagnosis, most caregivers will hold a negative attitude toward that healthcare provider (71). This issue is multiplied when considering rarer conditions. Often with limited exposure to FXS or genetic disorders, non-specialist healthcare providers have been shown to have limited knowledge about genetic testing in general as well as FXS (72, 73).

Knowledge Transfer to Improve Coherence of Mental Model

As mentioned above, mental models can evolve over time due to a growth in health literacy. This happens in large part due to knowledge transfer between caregivers and health professionals (48). While mental models are updated by knowledge transfer, knowledge transfer is also influenced by mental models. Indeed, researchers explained that when mental models of a specific topic do not align, knowledge transfer becomes challenging as "neither group can conceptualize the others' viewpoint" (48). During the knowledge transfer process, it is important to use terminology known and understood by families (74, 75). A successful knowledge transfer process involves information to be collected, evaluated, and organized, then shared using relevant language.

When caregivers cannot accurately communicate in a language understood by healthcare professionals, healthcare professionals struggle to fully understand how they can make an impact on the care of their patients and the lives of their caregivers (13). For healthcare professionals, it is important to assess the emotional state of the caregivers. For example, families having just received a diagnosis of FXS for their child identify this as a very distressing time (76). Inefficient knowledge transfer will often lead caregivers to be unsure when to seek help for their

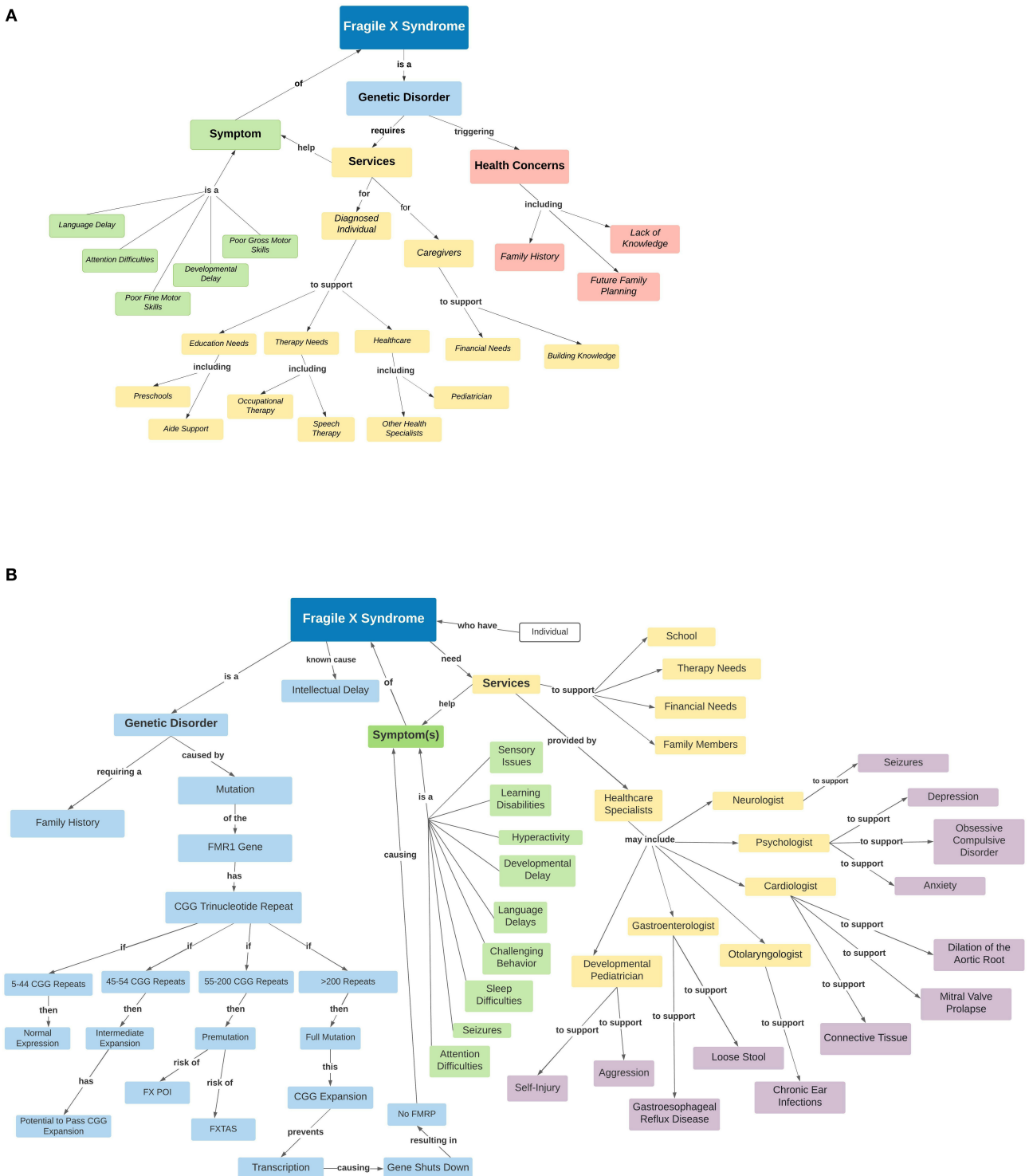

FIGURE 1 | Mental models vary between caregivers and healthcare professionals supporting individuals living with FXS. Concept maps representing the mental models of Fragile X syndrome symptoms and associated conditions (comorbidities) from a caregiver's perspective **(A)** and a healthcare professional's perspective **(B)**. This visual representation shows how mental models of FXS can vary between caregivers and health professionals, and may overlap in others (healthcare for instance). **(A)** The mental model of a caregiver may include concepts related to personal concerns outside of healthcare and a general understanding of the health system. **(B)** On the other hand, the mental model of a healthcare professional may only focus on the concepts related to healthcare more specifically, with a bias toward the consideration of their specialty. Legend of abbreviations used in the figure: FMR1, Fragile X mental retardation 1 gene; FXPOI, Fragile x Premature ovarian insufficiency; FXTAS, Fragile X tremor and ataxia syndrome.

child, to have feelings of inadequacy, to lack awareness of their child's condition, to have repeated interactions with healthcare professionals, to be unable to make decisions, and to be unaware of who to reach out to for care (13).

Knowledge Mapping: A Way to Represent and Share Complex Information Visually

Due to the complex, dynamic, and often incomplete information being shared between caregivers and healthcare professionals, finding an efficient way to present and organize communication is essential to quality care. Fortunately, cases in other fields have shown that knowledge mapping is a valuable tool to efficiently exchange concepts, as well as simplify verbal and written communication (77). In general, knowledge mapping is a tool used to support quality communication by visually representing information (78, 79). In our research we focused on using knowledge mapping to represent individuals' knowledge, beliefs, and perspectives. Several mapping methods are available and can be used in multiple applications depending on the need. These include: service blueprint, customer journey map, experience/care map, concept map or mental model diagram, and spatial map (80). We focused on two types of mapping tools: **(A)** concept map and **(B)** care map as they have been used most frequently to represent mental model and clinical care, respectively.

Concept Map

A concept map can visually represent the mental model of an individual by showing the relationships between concepts and ideas in a hierarchical manner (78). Concept maps link core concepts to related concepts (48). In **Figure 1** we illustrate an example of using concept maps to represent a sample mental model of FXS for a caregiver and healthcare professional. When comparing the concept map of the caregiver and the healthcare professional it is clear where the mental models of care needed to support an individual with FXS differ. Concept maps may constitute an initial step in communication between caregivers and health professionals, allowing each "team player" to express their perceived understanding of a condition. When communicating about the needs of an individual diagnosed with NDD, including FXS, acknowledging the diverse mental models of each care provider is essential as there is an important variation between individuals in the severity of not only core symptoms, but also associated conditions (comorbidities) as mentioned above.

Care Map

A care map is adapted from the principles of a mind map, which is a more general map of concepts related to a topic (78, 81). These maps are designed in a radial structure with the patient at the center and peripheral topics related to the individual's care needs for whom the map is made. Pictures and/or words are used to organically represent a topic. A care map for an individual with FXS shows the multiple and diverse needs (related to all comorbidities) in their life (example in **Figure 2**). As can be seen by comparing **Figure 2** to **Figure 1**, the care map is more applied and actionable than the concept map and shows how different service providers may overlap from different sectors (school, hospital, outpatient clinic, etc.). As such, we propose that the care map may be used downstream of the concept map in the optimal management of a patient. As Gavin points out, a picture is worth more than a thousand words, indicating that the visual of a care map delivers a powerful message of not only the complexities in care, but draws out the privilege, or lack thereof, some families may experience within the healthcare system (82). One family's map may identify multiple systems of care and services that may not be accessible to others due to location, knowledge of the services, diagnosis of the child, finances, language, or citizenship.

Benefit of Knowledge Mapping in Complex Medical Situations

Research on care coordination correlates the use of: (**A**) concept mapping and (**B**) care mapping to an increased level of communication between healthcare providers and caregivers, as well as between healthcare providers themselves (19, 74, 75, 83). Furthermore, in healthcare mapping can promote health literacy, build and organize an individual's mental model, and enhance knowledge transfer (78, 84).

Impact of Concept Mapping

Concept maps are crucial as they allow identification of blind spots between caregivers and health professionals involved. This is important as increasing healthcare specialization, but also diversity in caregiver perspectives leads to further differences in mental models (85). Concept maps, by putting individual mental models in the open, allow people to acknowledge differences in perspective, personal bias, and lack of awareness in some aspects of a concept which are more remote to one's experience. Concept maps have been shown to be core to efficient knowledge sharing (86), education, and enhanced problem solving (87).

Mental model representation with concept maps allows researchers to group results and provide higher order analysis (48). We also propose that teaching programs for both caregivers and healthcare professionals, as well as health policies and community resources, would be made more impactful by considering mental models.

Impact of Care Mapping

There are multiple advantages to using a care map as well. Care maps increase the level of communication between caregivers and healthcare providers, as well as between healthcare providers themselves, by increasing the level of engagement and reciprocal communication while also reducing the barriers in status (19, 74, 75). Care maps have the potential to give families a voice to express not only their child's needs, but their needs as a caregiver (19, 74, 75). This enhanced engagement also comes from developing a care map (17) and the empowerment of families by giving them an opportunity to share their voice and express preference and priorities around their child's needs and care concerns, hopes, and caregiver goals (19).

A care map can incorporate pictograms (**Figure 2**) having the potential to involve children in their care and provides the opportunity to prioritize the interventions that are most important to them. According to Adams et al., care mapping

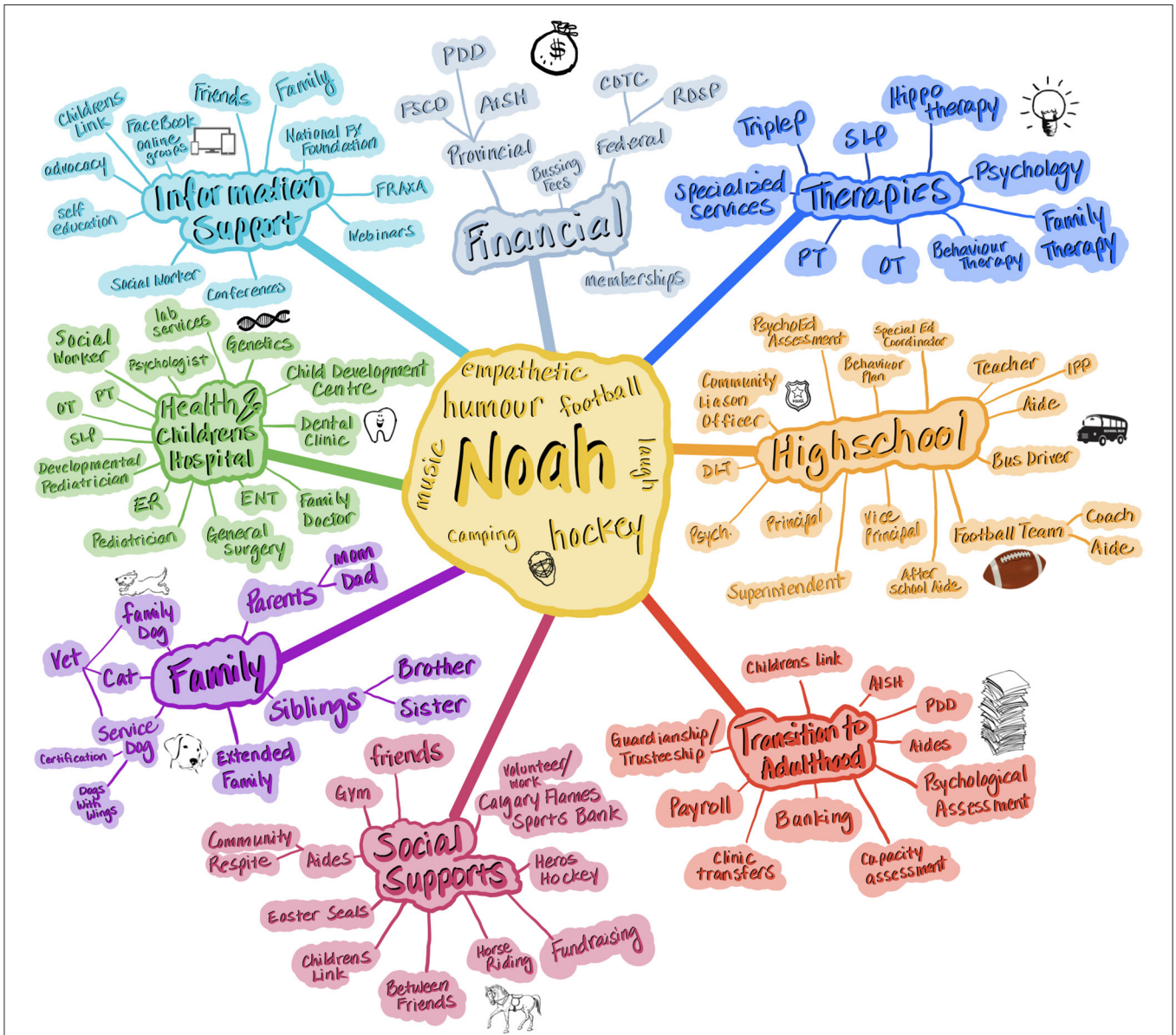

FIGURE 2 | Care maps help to display the multiple entities related to individuals with FXS. A care map captures the multidimensional aspect of care needs and is organized around a given individual. Use of pictures, colors, and shapes can help to involve youth in the development of the care map while identifying priorities over time. Care maps are often color coded to provide visual distinction between fields. Note that overlap between providers becomes more apparent and allows for collaboration. For instance, occupational therapy could be provided in school and community. Legend of terms used in the figure: PDD, Persons With Developmental Disabilities (Provincial funding program in Canada); FSCD, Family Support for Children with Disabilities (Provincial funding program in Canada); AISH, Assured Income for the Severely Handicapped (Provincial funding program in Canada); CDTC, Child Disability Tax Credit (Federal funding program in Canada); RDSP, Registered Disability Savings Plan; SLP, Speech Language Pathologist; PT, Physical Therapy; OT, Occupational Therapy; ER, Emergency Room; ENT, Ear, Nose, and Throat Specialist; IPP, Individual Performance Plan; DLT, Diverse Learning Teacher.

can be seen as a universal language between healthcare providers (19). A care provider does not need to know a patient personally to understand the meaning and information reflected in a care map. In addition, there are educational opportunities offered by evaluating the information presented in a care map (16).

Care mapping identifies the importance of forming relationships between all participants in the decision-making process (48) and is an effective tool to develop empathetic relationships within a limited time frame, build partnerships, and support collaboration (19).

Care mapping provides the opportunity for individuals to expand their existing knowledge by providing new ideas and viewpoints, in turn enhancing their mental models (78).

DISCUSSION

Our literature review investigated the challenges of communication between caregivers and healthcare providers caring for individuals living with complex health needs, including NDD and more specifically FXS. We found that one's understanding of the condition of their child/patient, referred to as the mental model, was key in driving decisions, actions, and communication. We showed how mental models vary over time between individuals, and how health literacy and knowledge transfer contribute to leveling those differences in mental models.

We then discussed knowledge mapping methods which could be used to better communicate information between healthcare providers and caregivers. We propose that different knowledge mapping methods be combined considering their different outputs. We suggest that concept maps be used as a tool to represent one's mental model of a given topic. We showed that concept maps can identify a shared understanding and limit misunderstanding. We discussed multiple case studies that identified how crucial it is to recognize individual mental models, not only to facilitate quality communication but also for compatible team functioning. Next, we discussed how the complex care team caring for individuals with NDD and FXS could be better coordinated when presented with a visual representation of information like a care map. We also highlighted how developing the care map together with healthcare professionals generated engagement and empowerment for caregivers.

Further studies will be required to assess the cost effectiveness of these different mapping methods in the context of NDD. Moreover, while in theory very useful, we noted that most research on mapping was performed in specialized clinical or even research environments, which are not standard for most individuals with NDD. We therefore wonder about the uptake of care mapping and concept maps in clinical setups beyond specialized programs. Furthermore, studies investigating the internal and external validity of care and concept maps will be important to determine their effectiveness across patients, or conditions, or stages of life for individuals with NDD. Finally, considering the important changes over the lifespan of individuals with NDD, the changes in literacy for caregivers and health professionals over time, it remains unclear how long a map will be useful before becoming in itself a barrier to communication. More research into when and how maps will need to be updated will have to be done in the future.

Altogether, we hope our review will generate interest into developing mapping approaches to improve communication between healthcare providers and caregivers but also for any individual involved in each individual's team.

AUTHOR CONTRIBUTIONS

KK and FB performed the literature review, co-designed the project, and co-wrote the manuscript. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Arora NK, Nair MKC, Gulati S, Deshmukh V, Mohapatra A, Mishra D, et al. Neurodevelopmental disorders in children aged 2-9 years: population-based burden estimates across five regions in India. *PLoS Med*. (2018) 15:e1002615. doi: 10.1371/journal.pmed.1002615
2. Emerson E. Deprivation, ethnicity and the prevalence of intellectual and developmental disabilities. *J Epidemiol Community Health*. (2012) 66:218–24. doi: 10.1136/jech.2010.111773
3. Taylor E. Developing ADHD. *J Child Psychol Psychiatry*. (2009) 50:126–32. doi: 10.1111/j.1469-7610.2008.01999.x
4. Johnson S, Fawke J, Hennessy E, Rowell V, Thomas S, Wolke D, et al. Neurodevelopmental disability through 11 years of age in children born before 26 weeks of gestation. *Pediatrics*. (2009) 124:e249–57. doi: 10.1542/peds.2008-3743
5. Zauche LH, Darcy Mahoney AE, Higgins MK. Predictors of Co-occurring neurodevelopmental disabilities in children with autism spectrum disorders. *J Pediatr Nurs*. (2017) 35:113–9. doi: 10.1016/j.pedn.2017.04.002
6. Hansen BH, Oerbeck B, Skirbekk B, Petrovski BÉ, Kristensen H. Neurodevelopmental disorders: prevalence and comorbidity in children referred to mental health services. *Nord J Psychiatry*. (2018) 72:285–91. doi: 10.1080/08039488.2018.1444087
7. Tatishvili N, Gabunia M, Laliani N, Tatishvili S. Epidemiology of neurodevelopmental disorders in 2 years old Georgian children. Pilot study – population based prospective study in a randomly chosen sample. *Eur J Paediatr Neurol*. (2010) 14:247–52. doi: 10.1016/j.ejpn.2009.07.004
8. Liddle M, Birkett K, Bonjour A, Risma K. A collaborative approach to improving health care for children with developmental disabilities. *Pediatrics*. (2018) 142:e20181136. doi: 10.1542/peds.2018-1136
9. Stille CJ. Communication, comanagement, and collaborative care for children and youth with special healthcare needs. *Pediatr Ann*. (2009) 38:498–504. doi: 10.3928/00904481-20090820-09
10. Forrest CB, Glade GB, Baker AE, Bocian A, von Schrader S, Starfield B. Coordination of specialty referrals and physician satisfaction with referral care. *Arch Pediatr Adolesc Med*. (2000) 154:499. doi: 10.1001/archpedi.154.5.499
11. Sinclair S, Kondejewski J, Schulte F, Letourneau N, Kuhn S, Raffin-Bouchal S, et al. Compassion in pediatric healthcare: a scoping review. *J Pediatr Nurs*. (2020) 51:57–66. doi: 10.1016/j.pedn.2019.12.009

12. Molina EG. Complex chronic conditions in pediatrics: needs for a level of complexity classification system. *Biomed J Sci Tech Res*. (2020) 31:24539–40. doi: 10.26717/bjstr.2020.31.005169
13. Altman L, Zurynski Y, Breen C, Hoffmann T, Woolfenden S. A qualitative study of health care providers' perceptions and experiences of working together to care for children with medical complexity (CMC). *BMC Health Serv Res*. (2018) 18:70. doi: 10.1186/s12913-018-2857-8
14. Courtney E, Kiernan G, Guerin S, Ryan K, McQuillan R. Mothers' perspectives of the experience and impact of caring for their child with a life-limiting neurodevelopmental disability. *Child Care Health Dev*. (2018) 44:704–10. doi: 10.1111/cch.12580
15. Miller JE, Nugent CN, Gaboda D, Russell LB. Reasons for unmet need for child and family health services among children with special health care needs with and without medical homes. *PLoS ONE*. (2013) 8:e82570. doi: 10.1371/journal.pone.0082570
16. Dewan T, Cohen E. Children with medical complexity in Canada. *Paediatr Child Health*. (2013) 18:518–22. doi: 10.1093/pch/18.10.518
17. Kuo DZ, McAllister JW, Rossignol L, Turchi RM, Stille CJ. Care coordination for children with medical complexity: whose care is it, anyway? *Pediatrics*. (2018) 141:S224–32. doi: 10.1542/peds.2017-1284g
18. Page BF, Hinton L, Harrop E, Vincent C. The challenges of caring for children who require complex medical care at home: 'The go between for everyone is the parent and as the parent that's an awful lot of responsibility'. *Health Expect*. (2020) 23:1144–54. doi: 10.1111/hex.13092
19. Adams S, Nicholas D, Mahant S, Weiser N, Kanani R, Boydell K, et al. Care maps for children with medical complexity. *Dev Med Child Neurol*. (2017) 59:1299–306. doi: 10.1111/dmcn.13576
20. Arim RG, Miller AR, Guèvremont A, Lach LM, Brehaut JC, Kohen DE. Children with neurodevelopmental disorders and disabilities: a population-based study of healthcare service utilization using administrative data. *Dev Med Child Neurol*. (2017) 59:1284–90. doi: 10.1111/dmcn.13557
21. Cohen E, Berry JG, Camacho X, Anderson G, Wodchis W, Guttmann A. Patterns and costs of health care use of children with medical complexity. *Pediatrics*. (2012) 130:e1463–70. doi: 10.1542/peds.2012-0175
22. Miller AR, Mâsse LC, Shen J, Schiariti V, Roxborough L. Diagnostic status, functional status and complexity among Canadian children with neurodevelopmental disorders and disabilities: a population-based study. *Disabil Rehabil*. (2013) 35:468–78. doi: 10.3109/09638288.2012.699580
23. McAllister JW, Presler E, Cooley WC. Practice-based care coordination: a medical home essential. *Pediatrics*. (2007) 120:e723–33. doi: 10.1542/peds.2006-1684
24. Antonelli RC, Mcallister JW, Popp J. *Making Care Coordination A Critical Component of the Pediatric Health System: A Multidisciplinary Framework*. (2009). Available online at: www.commonwealthfund.org (accessed March 27, 2021).
25. Androschuk A, Al-Jabri B, Bolduc FV. From learning to memory: what flies can tell us about intellectual disability treatment. *Front Psychiatry*. (2015) 6:85. doi: 10.3389/fpsyt.2015.00085
26. Brady NC, Fleming K, Bredin-Oja SL, Fielding-Gebhardt H, Warren SF. Language development from early childhood to adolescence in youths with fragile X syndrome. *J Speech Lang Hear Res*. (2020) 63:3727–42. doi: 10.1044/2020_JSLHR-20-00198
27. Fielding-Gebhardt H, Warren SF, Brady NC. Developmental trajectory of communication repair in children with fragile X syndrome. *Autism Dev Lang Impair*. (2020) 29:133–48. doi: 10.1177/2396941520909014
28. Haebig E, Sterling A, Barton-Hulsey A, Friedman L. Rates and predictors of co-occurring autism spectrum disorder in boys with fragile X syndrome. *Autism Dev Lang Impairments*. (2020) 5:1–19. doi: 10.1177/2396941520905328
29. Hagerman R, Hoem G, Hagerman P. Fragile X and autism: intertwined at the molecular level leading to targeted treatments. *Mol Autism*. (2010) 1:12. doi: 10.1186/2040-2392-1-12
30. Hagerman RJ, Narcisa V, Hagerman PJ. Chapter 46: Fragile X: a molecular and treatment model for autism spectrum disorders. In: Amaral D, Geschwind D, Dawson G, editors. *Autism Spectrum Disorders*. Oxford University Press (2011). p. 801–11. doi: 10.1093/med/9780195371826.003.0052
31. Rodriguez-Revenga L, Madrigal I, Mila M. Prevalence studies on fragile X alleles in autism. *Compr Guide Autism*. (2014) 2755–71. doi: 10.1007/978-1-4614-4788-7_185
32. Dueck A, Reis O, Bastian M, van Treeck L, Weirich S, Haessler F, et al. Feasibility of a complex setting for assessing sleep and circadian rhythmicity in a fragile X cohort. *Front Psychiatry*. (2020) 11:361. doi: 10.3389/fpsyt.2020.00361
33. Wirojanan J, Jacquemont S, Diaz R, Bacalman S, Anders TF, Hagerman RJ, et al. The efficacy of melatonin for sleep problems in children with autism, fragile X syndrome, or autism and fragile X syndrome. *J Clin Sleep Med*. (2009) 5:145–50. doi: 10.5664/jcsm.27443
34. Curran C, Debbarma S, Sedky K. Fragile X and obstructive sleep apnea syndrome: case presentation and management challenges. *J Clin Sleep Med*. (2017) 13:137–8. doi: 10.5664/jcsm.6408
35. Muller K, Brady NC, Warren SF, Fleming KK. Mothers' perspectives on challenging behaviours in their children with fragile X syndrome. *J Intellect Dev Disabil*. (2019) 44:481–91. doi: 10.3109/13668250.2018.1496379
36. Miller JG, Bartholomay KL, Lee CH, Bruno JL, Lightbody AA, Reiss AL. Empathy and anxiety in young girls with fragile X syndrome. *J Autism Dev Disord*. (2021). doi: 10.1007/s10803-021-05105-6
37. Schneider A, Seritan A, Tassone F, Rivera SM, Hagerman R, Hessl D. Psychiatric features in high-functioning adult brothers with fragile X spectrum disorders. *Prim Care Companion CNS Disord*. (2013) 15:PCC.12l01492. doi: 10.4088/pcc.12l01492
38. Gauthier-Boudreault C, Gallagher F, Trépanier J, Corbin F, Couture M. Factors impacting the transition to adulthood of youth with fragile X syndrome and their families: facilitators, obstacles and needs. *Res Dev Disabil*. (2020) 103:103674. doi: 10.1016/j.ridd.2020.103674
39. Hagerman RJ, Berry-Kravis E, Hazlett HC, Bailey DB Jr, Moine H, Kooy RF, et al. Fragile X syndrome. *Nat Rev Dis Primers*. (2017) 3:17065. doi: 10.1038/nrdp.2017.65
40. Van Remmerden MC, Hoogland L, Mous SE, Dierckx B, Coesmans M, Moll HA, et al. Growing up with fragile X syndrome: concerns and care needs of young adult patients and their parents. *J Autism Dev Disord*. (2020) 50:2174–87. doi: 10.1007/s10803-019-03973-7
41. Wheeler AC, Wylie A, Villagomez A, Bishop E, Raspa M. Health care for individuals with fragile X syndrome: understanding access and quality. *Disabil Health J*. (2019) 12:269–77. doi: 10.1016/j.dhjo.2018.11.004
42. Dew-Hughes D. *Educating Children with Fragile X Syndrome: A Multi-Professional View*. London; New York, NY: Routledge (2003).
43. Hay DA. Does IQ decline with age in fragile-X? a methodological critique. *Am J Med Genet*. (1994) 51:358–63. doi: 10.1002/ajmg.1320510412
44. Rootman I, Gordon-El-Bihbety D. A vision for a health literate Canada: report of the Expert Panel on Health Literacy [Internet]. *Canadian Public Health Association*; (2008). Available online at: https://search-ebscohost-com.login.ezproxy.library.ualberta.ca/login.aspx?direct=true&db=cat03710a&AN=alb.9307442&site=eds-live&scope=site (11) (accessed October 29, 2021).
45. Fielding-Gebhardt H, Warren SF, Brady NC. Child challenging behavior influences maternal mental health and relationship quality over time in fragile X syndrome. *J Autism Dev Disord*. (2020) 50:779–97. doi: 10.1007/s10803-019-04308-2
46. Sterling A, Warren SF. Parenting of children with down syndrome compared to fragile X syndrome. *Dev Neurorehabil*. (2018) 21:64–7. doi: 10.1080/17518423.2016.1259274
47. Fowler J. *The Power Of Mental Models: How To Make Intelligent Decisions, Gain A Mental Edge And Increase Productivity* (2019). San Francisco: Independently published.
48. Sturgiss E, Luig T, Campbell-Scherer DL, Lewanczuk R, Green LA. Using concept maps to compare obesity knowledge between policy makers and primary care researchers in Canada. *BMC Res Notes*. (2019) 12:23. doi: 10.1186/s13104-018-4042-x
49. Johnson-Laird PN. *Mental Models*. Cambridge: Cambridge University Press (1983).
50. McComb S, Simpson V. The concept of shared mental models in healthcare collaboration. *J Adv Nurs*. (2014) 70:1479–88. doi: 10.1111/jan.12307
51. Drach-Zahavy A, Broyer C, Dagan E. Similarity and accuracy of mental models formed during nursing handovers: a concept mapping approach. *Int J Nurs Stud*. (2017) 74:24–33. doi: 10.1016/j.ijnurstu.2017.05.009

52. Markin NW. Navigating to the Goal: the importance of shared mental models in complex environments. *J Cardiothorac Vasc Anesth*. (2018) 32:2618–9. doi: 10.1053/j.jvca.2018.03.019
53. D'Ambruoso SF, Coscarelli A, Hurvitz S, Wenger N, Coniglio D, Donaldson D, et al. Use of a shared mental model by a team composed of oncology, palliative care, and supportive care clinicians to facilitate shared decision making in a patient with advanced cancer. *J Oncol Pract*. (2016) 12:1039–45. doi: 10.1200/JOP.2016.013722
54. Sajatovic M, Davies M, Bauer MS, McBride L, Hays RW, Safavi R, et al. Attitudes regarding the collaborative practice model and treatment adherence among individuals with bipolar disorder. *Compr Psychiatry*. (2005) 46:272–7. doi: 10.1016/j.comppsych.2004.10.007
55. Power MJ. Psychological approaches to bipolar disorders: a theoretical critique. *Clin Psychol Rev*. (2005) 25:1101–22. doi: 10.1016/j.cpr.2005.06.008
56. Carnelley KB, Pietromonacó PR, Jaffe K. Depression, working models of others, and relationship functioning. *J Pers Soc Psychol*. (1994) 66:127–40. doi: 10.1037/0022-3514.66.1.127
57. Teasdale JD. Emotional processing, three modes of mind and the prevention of relapse in depression. *Behav Res Ther*. (1999) 37(Suppl. 1):S53–77. doi: 10.1016/S0005-7967(99)00050-9
58. von Hecker U, Sedek G. Uncontrollability, depression, and the construction of mental models. *J Pers Soc Psychol*. (1999) 77:833–50. doi: 10.1037/0022-3514.77.4.833
59. Pélissier MC, O'Connor KP, Dupuis G. When doubting begins: exploring inductive reasoning in obsessive-compulsive disorder. *J Behav Ther Exp Psychiatry*. (2009) 40:39–49. doi: 10.1016/j.jbtep.2008.03.003
60. Goodwin J, Schoch K, Shashi V, Hooper SR, Morad O, Zalevsky M, et al. A tale worth telling: the impact of the diagnosis experience on disclosure of genetic disorders. *J Intellect Disabil Res*. (2015) 59:474–86. doi: 10.1111/jir.12151
61. Nazareth T, Li N, Marynchenko M, Zhou Z, Chopra P, Signorovitch J, et al. Burden of illness among patients with Fragile X Syndrome (FXS): a medicaid perspective. *Curr Med Res Opin*. (2016) 32:405–16. doi: 10.1185/03007995.2015.1119678
62. Turbitt E, Klein WMP, Rogers K, D'Amanda CS, Biesecker BB. Parental decision making about clinical trial enrollment: a survey of parents of children with fragile X syndrome. *Health Psychol*. (2020) 39:1070–7. doi: 10.1037/hea0001038
63. Reines V, Charen K, Rosser T, Eisen A, Sherman SL, Visootsak J. Parental perspectives on pharmacological clinical trials: a qualitative study in down syndrome and fragile X syndrome. *J Genet Couns*. (2017) 26:1333–40. doi: 10.1007/s10897-017-0111-x
64. Mancuso JM. Assessment and measurement of health literacy: an integrative review of the literature. *Nurs Health Sci*. (2009) 11:77–89. doi: 10.1111/j.1442-2018.2008.00408.x
65. Mårtensson L, Hensing G. Health literacy - a heterogeneous phenomenon: a literature review. *Scand J Caring Sci*. (2012) 26:151–60. doi: 10.1111/j.1471-6712.2011.00900.x
66. Murphy NA, Darro N. Parents of children with medical complexity are essential health care personnel. *Pediatrics*. (2021) 147:e2021050160. doi: 10.1542/peds.2021-050160
67. Lawrence PR, Feinberg I, Spratling R. The relationship of parental health literacy to health outcomes of children with medical complexity. *J Pediatr Nurs*. (2021) 60:65–70. doi: 10.1016/j.pedn.2021.02.014
68. Palfrey J, Gasser U. *The Connected Parent: An Expert Guide to Parenting in a Digital World*. New York, NY: Hachette (2020).
69. Whitley JA, Kieran K. Accuracy and comprehensiveness of publicly-available online data about bedwetting: an actionable opportunity to improve parent and caregiver self-education. *J Pediatr Urol*. (2020) 16:661.e1–8. doi: 10.1016/j.jpurol.2020.07.013
70. Smolich L, Charen K, Sherman SL. Health knowledge of women with a fragile X premutation: improving understanding with targeted educational material. *J Genet Couns*. (2020) 29:983–91. doi: 10.1002/jgc4.1222
71. Minnes P, Steiner K. Parent views on enhancing the quality of health care for their children with fragile X syndrome, autism or down syndrome. *Child Care Health Dev*. (2009) 35:250–6. doi: 10.1111/j.1365-2214.2008.00931.x
72. Lindsay P. *Care of the Adult with Intellectual Disability in Primary Care*. Boca Raton, FL: CRC Press (2016).
73. Savatt JM, Myers SM. Genetic testing in neurodevelopmental disorders. *Front Pediatr*. (2021) 9:526779. doi: 10.3389/fped.2021.526779
74. Antonelli RC, Mcallister JW, Popp J. *Making Care Coordination a Critical Component of the Pediatric Health System: A Multidisciplinary Framework*. (2009). Available online at: www.commonwealthfund.org (accessed March 27, 2021).
75. Desai AD, Wang G, Wignall J, Kinard D, Singh V, Adams S, et al. User-centered design of a longitudinal care plan for children with medical complexity. *J Am Med Inform Assoc*. (2020) 27:1860–70. doi: 10.1093/jamia/ocaa193
76. Carmichael B, Pembrey M, Turner G, Barnicoat A. Diagnosis of fragile-X syndrome: the experiences of parents. *J Intellect Disabil Res*. (1999) 43:47–53. doi: 10.1046/j.1365-2788.1999.43120157.x
77. Ogden K, Kilpatrick S, Elmer S, Rooney K. Attributes and generic competencies required of doctors: findings from a participatory concept mapping study. *BMC Health Serv Res*. (2021) 21:560. doi: 10.1186/s12913-021-06519-9
78. Davies M. Concept mapping, mind mapping and argument mapping: what are the differences and do they matter? *High Educ*. (2011) 62:279–301. doi: 10.1007/s10734-010-9387-6
79. Schmehl P. *Introduction to Concept Mapping in Nursing*. Burlington, MA: Jones & Bartlett Publishers (2013).
80. Kalbach J. *Mapping Experiences: A Complete Guide to Creating Value through Journeys, Blueprints, and Diagrams*. Sebastopol, CA: O'Reilly Media, Inc. (2016).
81. Park SA, Miller DS, Boorman ED. Protocol for building a cognitive map of structural knowledge in humans by integrating abstract relationships from separate experiences. *STAR Protoc*. (2021) 2:100423. doi: 10.1016/j.xpro.2021.100423
82. Gavin F. An imaginative partnership: parents and the doctors who care for their children. *Paediatr Child Health*. (2009) 14:295–7. doi: 10.1093/pch/14.5.295
83. Atay S, Karabacak U. Care plans using concept maps and their effects on the critical thinking dispositions of nursing students. *Int J Nurs Pract*. (2012) 18:233–9. doi: 10.1111/j.1440-172X.2012.02034.x
84. Schuster PM. *Concept Mapping: A Critical-Thinking Approach to Care Planning*. Winnipeg, MB: F.A. Davis Co (2016).
85. Street F. *The Great Mental Models: General Thinking Concepts*. San Diego: Latticework Publishing Inc. (2019).
86. Hussain H, Shamsuar NR. Concept map in knowledge sharing model. *Int J Inf Educ Technol*. (2013) 3:397–400. doi: 10.7763/ijiet.2013.v3.306
87. Karadag M, Demir SG, Bulut H, Erden S, Demir N, Erdogan Z, Ay A. The effect of concept map based education on the problem solving skills of students. In: *8th World Conference on Educational Sciences (WCES-2016)*. Madrid (2017). p. 506–13. Available online at: www.prosoc.eu

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Delineating the Relationships Between Motor, Cognitive-Executive and Psychiatric Symptoms in Female *FMR1* Premutation Carriers

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Introduction: Premutation expansions (55–200 CGG repeats) of the Fragile X Mental Retardation 1 (FMR1) gene on the X chromosome are associated with a range of clinical features. Apart from the most severe - Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS) - where the most typical white matter changes affect cerebellar peduncles, more subtle changes may include impairment of executive functioning, affective disorders and/or subtle motor changes. Here we aimed to examine whether performance in selected components of executive functioning is associated with subclinical psychiatric symptoms in non-FXTAS, adult females carrying the *FMR1* premutation.

Methods and Sample: A total of 47 female premutation carriers (sub-symptomatic for FXTAS) of wide age range (26–77 years; M = 50.3; SD = 10.9) were assessed using standard neuropsychological tests, three motor rating scales and self-reported measures of psychiatric symptoms using the Symptom Checklist-90-Revised (SCL-90-R).

Results: After adjusting for age and educational level where appropriate, both non-verbal reasoning and response inhibition as assessed on the Stroop task (i.e., the ability to resolve cognitive interference) were associated with a range of primary psychiatric symptom dimensions, and response inhibition uniquely predicted some primary symptoms and global psychiatric features. Importantly, lower scores (worse performance) in response inhibition were also strongly correlated with higher (worse) scores on standard motor rating scales for tremor-ataxia and for parkinsonism.

Conclusion: These results provide evidence for the importance of response inhibition in the manifestation of psychiatric symptoms and subtle tremor-ataxia motor features, suggestive of the presence of early cerebellar changes in female premutation carriers.

Keywords: fragile X premutation, response inhibition, executive function, psychiatric symptoms, ataxia, cerebellum, motor, Fragile X-Associated Tremor/Ataxia Syndrome (FXTAS)

INTRODUCTION

The premutation expansion (PM: 55–200 CGG repeats) of the Fragile X Mental Retardation 1 (*FMR1*) gene on the X chromosome is associated with a range of clinical features, including executive function impairments and affective disorders (1). The most severe form of clinical disorder associated with premutation expansions in the 5′ untranslated region of the Fragile X Mental Retardation 1 (*FMR1*) gene is a late-onset progressive neurodegenerative condition: Fragile X-Associated Tremor/Ataxia Syndrome (2). The clinical features include intention and/or postural tremor, gait ataxia, dementia, and in some cases parkinsonism. This syndrome usually occurs after, and progresses from, the age of 55 years, and is more prevalent in male (45%) than in female (8–16%) PM carriers. In addition to FXTAS, women carrying the PM allele are at increased risk (∼20%) of developing premature ovarian failure (3). Apart from these two well-recognized disorders, subtle cognitive-executive and neuropsychiatric features are not uncommon (4), and the latter have more recently been recognized as a distinct group termed: Fragile X-Associated Neuropsychiatric Disorders (FXAND) (5). These include cognitive-executive impairments, anxiety, social deficits, depression and obsessive compulsive features.

Although the presence of a cognitive-executive phenotype in female PM carriers has been controversial in the literature, deficits have been reported in the area of response inhibition, using direct behavioral assessment and oculomotor antisaccade paradigms (6–9). Response inhibition is an aspect of executive function that enables suppression of a prepotent response that is inappropriate in a given context (10). Several studies documented age-dependent changes in response inhibition deficits in female PM carriers, with older age associated with slower inhibition of prepotent verbal responses and longer antisaccade latency (6, 8). However, some of these findings reported in the literature have been inconsistent (11, 12), perhaps owing to selection bias, choice of response inhibition measure, discrepant age ranges, and small sample sizes.

Apart from cognitive-executive issues, the effect of the PM alleles has been documented in elevated prevalence of some psychiatric disorders, especially mood and anxiety disorders (4), with elevated symptoms of social phobia, obsessive-compulsive behavior, somatization and anxiety/depression, in female PM carriers with or without the environmental stressors arising from raising a child with Fragile X syndrome (4, 13, 14). With regard to trajectories in female PM carriers, there is evidence for increasing deterioration of psychiatric problems especially in anxiety and obsessive-compulsive symptoms (15), and increasing prevalence and severity of mood and anxiety disorders over time (4). Notably, poorer inhibition of verbal responses have been linked to self-reports of elevated anxiety and depression in non-FXTAS asymptomatic female PM carriers (7), suggesting that subtle cognitive-executive difficulties may contribute to or co-occur with psychiatric features.

Previous findings that impairments in a range of motor domains are associated with increasing CGG repeat size in otherwise unaffected PM carriers (16, 17) indicate that multiple sensorimotor issues correspond with increased disease risk prior to development of prominent symptoms and observable clinical signs. Several studies have reported subtle sensorimotor deficits in apparently asymptomatic (non-FXTAS) female PM carriers, including changes in upper limb manual control (18), step initiation (19), gait function, and postural control (20–22). Importantly, other studies have documented associations between gait, stepping/postural control and cognitive-executive functions in PM carriers with and without FXTAS (19–21).

Delineating the female PM carrier phenotype at multiple severities across motor, cognitive-executive, and psychiatric domains will be important for examination of synergistic effects of gender, aging and the *FMR1* premutation allele on these subclinical features.

In a recent study, we have shown that mild motor deficits (cerebellar ataxia and tremor scores) were correlated with set-shifting, working memory and psychomotor speed in female PM carriers without a diagnosis of FXTAS (23). Thus, there is converging evidence for the view that there is a continuum of subclinical changes that accumulate over the lifetime, irrespective of the final clinical endpoint. However, there has been no studies considering the inter-relationships between all three domains—motor, cognitive-executive and neuropsychiatric—in female PM carriers within the same study.

The present study explores the relationships between distinct subcomponents of cognitive-executive functioning and a range of psychiatric symptoms in a sample of non-FXTAS, clinically unaffected female PM carriers across a broad range of age and CGG expansion sizes. Based on previous literature, we tested the hypothesis that poorer inhibitory control will be correlated with higher levels of psychiatric symptoms in the absence of formal diagnoses. We have also considered a specific relationship between inhibitory control and subtle motor features of tremor-ataxia in female PM carriers. We hypothesized that worse motor rating scores will be correlated with reduced cognitive inhibition performance at a subclinical but detectable level in female PM carriers.

METHODS AND SAMPLE

Participants

The study protocol was approved by the La Trobe University and Monash University Human Research Ethics Committees and informed consent was provided by all participants. The sample comprised a total of 47 adult female PM carriers aged between 26 and 77 years (mean = 50.3; SD = 10.9, CGG repeats from 56 to 133), recruited through cascade testing of a large cohort of fragile X families as described in our previous studies (24–26). All participants were white Caucasian with the exception of one participant of Asian descent. The sample characteristics of the female PM carriers is provided in **Table 1**. On the basis of FXTAS criteria, none of the female PM carriers met diagnostic criteria for inclusion as FXTAS. Based on formal testing by two neurologists (ES & DZL), elevated scores on standard neurological motor rating scales (see below) were identified in 27 female PM carriers; however, we classified this increase as "sub-symptomatic for

TABLE 1 | Demographic, motor and cognitive-psychiatric characteristics.

				Normative values[a]	
Variable	**Mean**	**SD**	**Range**	**Mean**	**SD**
Characteristic					
Age	50.3	10.9	26–77		
Age of menopause	43.6	7.1	27–53		
Year of Education	12.9	3.3	4–22		
CGG repeats	82.7	16.5	56–133		
Motor Score					
UPDRS	2.5	3.3	0–17	1.9	2.0[b]
ICARS Total	6.7	3.6	2–16	4.1	2.2[c]
Cognitive measures					
Vocab SS	9.8	2.5	4–17	10.5	3.2[d]
MR SS	12.2	2.9	5–17	10.4	2.9[d]
DS Forwards	6.5	1.2	4–9	7.6	0.8[e]
DS Backwards	4.9	1.4	3–8	3.1	1.2[e]
Pro-rated IQ	103.6	13.2	74–132	–	–
TMT A (raw score)	35.8	13.3	17–105	31.8	9.9[f]
TMT B (raw score)	79.3	31.3	37–168	78.8	19.1[f]
TMT B-A	46.0	26.8	8–128	32.0	14.4[f]
Stroop (t-score)	51.9	9.2	34–71	43.9	23.1[g]
SDMT (raw score)	51.0	10.5	33–74	47.9	10.6[h]
SCL-90-R Scales (t scores)[i]					
Somatization	49.3	10.1	35–79	46.6	8.4
Depression	50.0	9.5	34–69	49.0	11.2
Interpersonal sensitivity	50.7	9.3	39–71	50.5	10.4
Phobic anxiety	47.2	6.7	41–66	46.9	8.3
Paranoid ideation	48.3	8.6	41–65	50.0	8.6
Obsessive-compulsive	51.7	10.0	37–74	51.7	9.3
Hostility	47.3	6.8	35–65	47.1	7.5
Anxiety	46.3	8.9	37–65	46.0	8.6
Global Severity Index (GSI)	48.7	11.3	30–69	–	–

[a] *Normative data derived from relevant studies where available.*
[b] *Postuma et al. (27).*
[c] *Fitzpatrick et al. (28).*
[d] *Harrison et al. (29).*
[e] *Leung et al. (30).*
[f] *Tombaugh (31).*
[g] *Bayard et al. (32).*
[h] *Kiely et al. (33).*
[i] *Gossett et al. (14).*
Vocab SS, WAIS-III Vocabulary Scaled Score; MR SS, WAIS-III Matrix Reasoning Scaled Score; DS Forwards, WAIS-III forwards digit span subtotal; DS Backwards, WAIS-III backwards digit span subtotal; TMT-A and -B, Trail-Making Test A and B; SDMT, Symbol-Digit Modalities Test.

FXTAS" based on standard testing using motor and cognitive scale scores as previously described (23).

Cognitive-Executive Measures

The cognitive measures were selected to focus on domains that relied on executive functioning, non-verbal reasoning and psychomotor speed, which are domains of impairment observed in male and female PM carriers [see (1) for a review]. General cognitive ability was assessed by the Vocabulary and Matrix Reasoning subtests of the Wechsler Adult Intelligence Scale (Third Edition; WAIS-III), which were used to calculate a prorated Full Scale IQ score (34). We used the Matrix Reasoning subtest as a measure of non-verbal reasoning. The WAIS-III Digit Span forward and backward components, assessed separately, were selected as measures of short-term verbal memory and working memory, respectively. Task or set switching was measured using the Trail Making Test (35). The Stroop Color-Word Test (36) was used as a measure of response inhibition in which the participant is asked to name the (ink-printed) color of a series of words of different colors (blue, green, red, brown, purple) while selectively inhibiting the automatic response of reading the names of printed color words aloud. For example, the word "blue" might be printed in green ink with the participant instructed to respond "green." Time to complete the task (typically <10 min for the Victoria version) was recorded and a decrease in color-naming speed was taken as measure of increased color-word interference. Finally, the Symbol Digit Modalities Test was used as a measure of psychomotor speed (37).

Motor Rating Scales

Tremor-ataxia motor signs were identified and scored using the International Cooperative Ataxia Rating Scale (ICARS) (38); and parkinsonian features using the Unified Parkinson's Disease Rating Scale (UPDRS) Part III-Motor (39). These standard neurological motor rating scales, with established inter-rater reliability (40–42) were administered and scored by two neurologists (ES & DZL) with relevant experience in administering these scales from previous studies.

Psychiatric Symptom Measures

The SCL-90-R is a 90 item self-report questionnaire measuring a range of symptom clusters and general psychiatric symptomatology occurring over the past week (43). The 90 items are clustered into nine primary symptom dimensions: somatization, obsessive compulsive, interpersonal sensitivity, depression, anxiety, hostility, phobic anxiety, paranoid ideation, and psychoticism. The Global Severity Index (GSI) is an overall measure of the level of psychological distress and intensity of symptoms, and is calculated from the average of the primary symptom scales (43). It has good internal consistency with coefficient alphas ranging from 0.77 to 0.90 across scales, and test-retest reliability over a period of 1 week from 0.80 to 0.90. We report T-scores for both the symptom dimension scales and the overall level of psychiatric disturbance (GSI T-score), with a score between 60 and 63 considered borderline and above 63 classified as above clinically significant threshold.

Genetic Molecular Measures

CGG Repeat Size

Standard methods were employed to isolate genomic DNA from peripheral blood lymphocytes using polymerase chain reaction and Southern blot analyses as described in our previous publications (23). Briefly, the Southern blot analysis involved digestion with 10 micrograms (μg) of isolated DNA using EcoRI and NruI. The *FMR1* genomic dig-labeled StB12.3 probe was

used for hybridization as previously described (44). PCR was used to amplify the genomic DNA (45).

Statistical Analysis

Data were analyzed using the Statistical Package for the Social Sciences version 26 (SPSS; IBM Corporation; Armonk, NY, USA). First, outliers were identified using boxplots, histograms, and by examining Z-scores. There were no extreme outliers removed from the dataset and standardized values were within the ranges −3.29 to 3.29. The distributions of scores for the motor measures and the cognitive measures were then evaluated for deviations from normality using Shapiro-Wilk's test of normality, disclosing some violations of the assumption of normality as required for parametric tests. Second, we conducted Spearman's rank correlation (ρ) to examine correlations between motor scores, cognitive-executive measures and psychiatric symptom scores, after adjusting for age and/or year of education (where appropriate). We then used the Holm-Bonferroni family-wise false discovery rate (FWFDR) correction method to correct the *p*-values for multiple comparisons.

The final approach consisted of a series of linear multiple regressions which were applied to identify the most significant predictors of psychiatric symptomatology on the SCL-90-R. This analysis was based on selected cognitive-executive variables found to be significantly related to psychiatric scores in correlational analysis. Age and years of education were entered as independent control variables. The assumptions of singularity and multicollinearity were met ($r < 0.7$), and inspection of residual scatterplots indicated assumptions of normality, linearity, homoscedasticity, and independence of residuals were also met. Cook's distances were less than one and values of the standardized residuals were between −3 and 3.

RESULTS

Sample Characteristics

Table 1 shows the sample characteristics for the cognitive-executive and SCL-90-R dimension scales and Global Severity Index. There was a single participant aged 26 years; the majority of the participants were aged between 35 and 50 years. CGG repeat length ranged from 56 to 133. We reported in a previous study (23) that the scores on the ICARS and the UPDRS were elevated in a proportion of the female carriers from the same sample as in the current study. However, we categorize this increase as sub-symptomatic for FXTAS since it had not generated any specific medical diagnoses, or realization of abnormality on the part of those individuals presenting with evidently abnormal scores.

As seen in **Table 1**, the scores on the cognitive measures were mostly comparable to normative values taken from relevant studies, with the exception of higher Stroop Color-Word Test t-scores in female PM carriers. On the SCL-90-R Global Severity Index, there were six participants who scored above the clinically significant threshold of 63, and another five participants scored in the borderline clinical range. However, the mean scores for premutation females on dimension scales of the SCL-90-R were comparable to control values as reported in Gossett et al. (14).

Correlations Between Motor, Cognitive and Psychiatric Symptom Scores

Spearman's rank correlations between cognitive-executive and SCL-90-R scores are provided in **Table 2**. There were significant negative correlations between Matrix Reasoning scaled scores (higher is better) and the Hostility symptom scale (higher is worse) ($p = 0.003$). Stroop Color-Word Test t-scores were significantly negatively correlated with scores on several of the SCL-90-R dimension scales (Depression, Hostility, Anxiety), and with the Global Severity Index (between $p = 0.019$–0.046). Considering the multitude of relationships of the Stroop Color-Word test with psychiatric symptom scales, we additionally explored the relationship of these scores with the motor scale scores and found that Stroop t-scores were significantly (negatively) correlated with both ICARS and UPDRS ($\rho = -0.50$, $p = 0.001$, with ICARS; $\rho = -0.44$, $p = 0.003$, with UPDRS). The scatterplots illustrating the nature of these relationships are shown in **Figure 1**.

Multiple Linear Regression Analysis for Cognitive-Executive Variables Predicting Psychiatric Symptom Scores

The multiple regression analysis showed that the Stroop Color-Word Test scores were a significant predictor of primary psychiatric symptom dimensions, Somatization ($\beta = -0.35$, $p = 0.049$) and Phobic Anxiety ($\beta = -0.34$, $p = 0.049$) (**Table 3**), and overall severity of psychiatric symptoms ($\beta = -0.33$, $p = 0.017$) on the SCL-90-R Global Severity Index (**Table 4**).

DISCUSSION

The present study is the first to consider relationships between all three phenotypic domains– motor, cognitive-executive and psychiatric features, in a sample of non-FXTAS adult female PM carriers. These relationships specifically involved cognitive components representing aspects of executive functioning, including the Stroop Word-Color test of inhibitory control and several psychiatric symptom domains as measured by the SCL-90-R. Here we also provide evidence that this important component of executive functioning—the ability to inhibit cognitive interference—is significantly (negatively) correlated with the motor scales representing tremor/gait ataxia (ICARS) and parkinsonism (UPDRS) that are commonly used to evaluate motor dysfunction in PM carriers.

Our earlier study, based on data from the same sample of female PM carriers, found that a range of cognitive measures, especially those targeting executive functioning and psychomotor speed, were significantly and consistently correlated with these two motor scale scores (23). Here we expanded these results by including a measure of response inhibition using the Stroop Color and Word test, as well as a range of psychiatric features on the SCL-90-R. We showed significant correlations between both the Stroop and the Matrix Reasoning scores, and a range of psychiatric features on the SCL-90-R. Together these results provide convincing evidence that all three components—motor, cognitive-executive and affective

TABLE 2 | Spearman's rank correlation (ρ) between cognitive-executive measures and SCL-90-R psychiatric symptoms, adjusted for age and/or year of education (whenever appropriate).

	MR SS[#]		DS forwards[#]		DS backwards		Stroop		TMT B-A[γ]		SDMT[γ]	
	ρ	*p*-value	ρ	*p*-value	ρ	*p*-value	ρ	*p*-value	ρ	*p*-value	ρ	*p*-value
Somatization	−0.06	0.715	0.00	0.999	−0.10	0.500	−0.29	0.050	−0.24	0.125	−0.02	0.895
Depression	−0.25	0.093	0.08	0.594	0.08	0.604	−0.37	**0.036***	−0.04	0.811	−0.09	0.582
Interpersonal	−0.22	0.155	−0.03	0.848	0.05	0.728	−0.20	0.183	−0.12	0.473	−0.06	0.723
Phobic anxiety	−0.05	0.723	−0.08	0.611	−0.18	0.241	−0.23	0.129	−0.01	0.998	−0.07	0.671
Paranoid ideation	−0.19	0.210	0.04	0.817	−0.06	0.690	−0.19	0.220	−0.08	0.629	−0.02	0.878
Obsessive-compulsive	−0.20	0.195	0.16	0.306	−0.04	0.786	−0.19	221	−0.14	0.420	−0.01	0.997
Hostility	−0.43	**0.003***	0.10	0.504	−0.21	0.159	−0.30	**0.046***	−0.14	0.396	−0.02	0.916
Anxiety	−0.20	0.179	0.01	0.970	−0.23	0.135	−0.31	**0.041***	−0.10	0.521	−0.05	0.768
Psychoticism	−0.19	0.222	0.09	0.540	0.01	0.939	−0.16	0.286	−0.05	0.744	−0.01	0.971
Global Severity Index	−0.28	0.065	0.02	0.973	0.03	0.831	−0.34	**0.019***	−0.15	0.362	−0.06	0.727

**p-values remain α = 0.05 after adjustment using the Holm-Bonferroni family-wise false discovery rate (FWFDR) correction method. Bolded p-values indicate that correlation is significant after FWFDR correction. [#]corrected for years of education.*

[γ] corrected for age and years of education.

Note there were significant correlations between Stroop Color-Word T Score and both ICARS (ρ = −0.503, p = 0.001) and UPDRS (ρ = −0.436, p = 0.003), after adjusting for age. MR SS, WAIS-III Matrix Reasoning Scaled Score; DS Forwards, WAIS-III forwards digit span subtotal; DS Backwards, WAIS-III backwards digit span subtotal; Stroop, Stroop Color-Word T Score; TMT B-A, Trail-Making Test time difference between B and A; SDMT, Symbol-Digit Modalities Test.

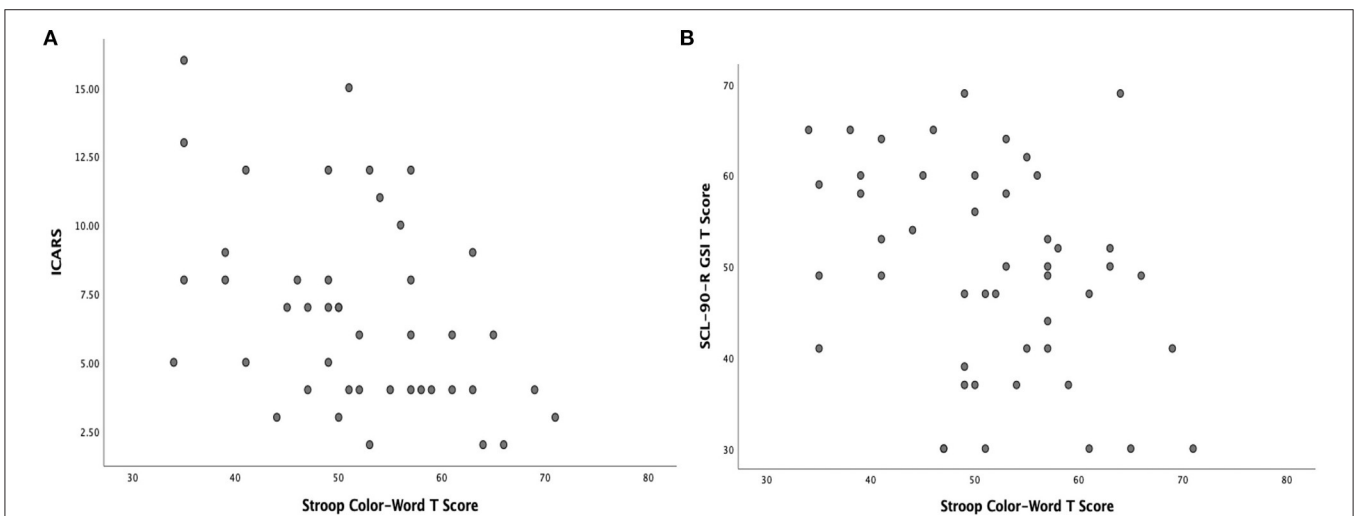

FIGURE 1 | (A) Scatterplots illustrating significant relationship between Stroop Color-Word t-score and ICARS Total score and **(B)** Scatterplots illustrating a significant relationship between Stroop Color-Word t-score and SCL-90-R Global Severity Index (GSI) t-score.

(psychiatric) manifestations—are interrelated at a subtle yet detectable level in female PM carriers. It is of special interest that such a constellation of salient relationships between features of motor, cognitive and affective abnormalities is remininscent in a milder form to the constellation of cognitive impairments (particularly affecting executive functions), affective changes and motor dysfunction, specifically attributed to cerebellar damage (46).

Although we did not have direct evidence of specific cerebellar pathology in our female PM carriers (which would require MRI or brain biopsy), and none of the participants manifested obvious clinical symptoms of cerebellar ataxia, the results of the two motor scales applied in this study showed that nearly half of the carriers had at least one of these scores increased beyond two standard deviations, which clearly reflects some degree of cerebellar dysfunction (23). It should be acknowledged that the motor rating scales especially the ICARS, recognize the symptoms which occur, albeit in much more severe form, in clinically diagnosable FXTAS, and are associated with white matter lesions predominantly found in the middle cerebellar peduncles (15).

The current findings highlight the importance of impairment of the ability to ignore interference, as measured by the Stroop Color and Word test, in predicting certain psychiatric features on the SCL-90-R. These findings are consistent with a previous study in female PM carriers showing that psychological

TABLE 3 | Multiple linear regression analyses for cognitive-executive variables predicting SCL-90-R symptom scale scores in female premutation carriers.

	Somatization				Phobic anxiety			
Predictor	***B***	***SE B***	***β***	***t***	***B***	***SE B***	***β***	***t***
Age	−0.149	0.150	−0.164	−0.992	−0.082	0.094	−0.144	−0.875
YOE[a]	−0.723	0.587	−0.218	−1.230	−0.581	0.367	−0.279	−1.582
MR SS[b]	0.584	0.688	0.158	0.849	0.755	0.430	0.325	1.754
Stroop[c]	−0.379	0.186	−0.351*	−2.034	−0.235	0.116	−0.346*	−2.017

[a] *Years of education.*
[b] *Matrix Reasoning Scaled Score.*
[c] *Stroop Color-Word Scaled Score. Standardized regression coefficients.*
*$p < 0.05$.

TABLE 4 | Multiple linear regression analyses for cognitive-executive variables predicting SCL-90-R Global Severity Index in female premutation carriers.

	Global Severity Index			
Predictor	***B***	***SE B***	***β***	***t***
Age	0.015	0.167	0.015	0.093
YOE[a]	−0.076	0.157	−0.069	−0.485
MR SS[b]	−0.148	0.171	−0.863	0.849
Stroop[c]	−0.129	0.049	−0.328*	−2.475

[a] *Years of education.*
[b] *Matrix Reasoning Scales Score.*
[c] *Stroop Color-Word Scaled Score.*
*Standardized regression coefficients. *$p < 0.05$.*

symptoms of anxiety and depression were highly elevated in those with poor response inhibition (7). Thus, the assessment of executive functions such as response inhibition could be useful in identifying risk factors for onset of significant psychiatric problems, especially anxiety and/or depression. Alternatively, these subtle executive function abnormalities may simply reflect the effects of psychological symptomatology on cognition. We may further speculate that subtle changes in the cerebellar peduncles in female PM carriers could give rise to mild cognitive regulatory impairments that interact with psychiatric symptomatology (perhaps via shared neuropathology) over time, and that in a subset of these carriers, may progress to a more severe clinical condition such as FXTAS. However, the possibility of the emergence and symptomatic expression of both cognitive inhibition impairments and psychiatric changes remains hypothetical, considering the cross-sectional nature of this study.

Given the multifaceted nature and fractionated components of executive functioning (47, 48), it is perhaps not surprising that some but not all cognitive-executive aspects might correlate with subtle motor features, as has been shown in our sample. The ability to suppress an unwanted response to avoid interference from prepotent stimuli, as assessed by the Stroop test, is commonly associated with recruitment of distinct regions in the ventromedial prefrontal cortex (49). However, we suggest that the cerebellum is likely to play an important role in inhibitory control by virtue of connectivity from medial prefrontal cortex through the pons and middle cerebellar peduncle via the cortico-ponto-cerebellar pathway (50). Although the presence of early cerebellar changes remains relatively unexplored in female PM carriers, there is evidence for subtle white matter alterations in the middle cerebellar peduncles in asymptomatic (non-FXTAS) male premutation carriers (51). In addition, the presence of abundant intranuclear inclusions has been reported throughout the brain in female PM carriers with and without FXTAS (52).

This study has a number of limitations. First, the reliance on self-reported psychological symptoms might be subject to biases and inaccuracy of participants' recall of their previous levels of psychological distress. Future studies applying semi-structured interviews will provide a more comprehensive evaluation; for example by using the Structured Clinical Interview for DSM-IV-TR (SCID) to confirm these relationships. Second, considering the variability of these measures, a larger sample size would provide more reliable results. Third, given the cross-sectional nature of this study, we could not assess the impact of executive function skills on psychiatric symptoms over time, and thus, were unable to determine whether the trajectories of any decline in motor function or cognition match those of any associated psychopathological impairments. Fourth, it should be acknowledged that the cohort of female PM carriers predominantly were mothers of children affected by FXS, hence our findings may not be generalizable to the broader population. Given the evidence of the effects of parenting stress of raising a child with a disability on increased risk for some psychiatric disorders (53, 54), future studies are needed in female PM carriers without children affected by FXS or other forms of disability. A final limitation is the lack of more sensitive and tract-based analysis of subclinical changes using advanced MRI techniques to examine relationships between microstructural alterations to the cerebellum and the executive-psychiatric phenotype in female PM carriers, but this preliminary observation may open the way for a future separate study.

In summary, our findings highlight the importance of response inhibition in the recognition of psychiatric symptoms and subtle tremor-ataxia motor features in female PM carriers

subsymptomatic for FXTAS. We report interactive influences across the constellation of features reminiscent in a milder form in disorders associated with disruption to the cerebro-cerebellar circuits. Our findings of the involvement of all three interacting domains of PM carrier phenotype, together with motor involvement (tremor and cerebellar ataxia), suggest that these early changes may result from the subtle impact of PM alleles on early changes in the cerebro-cerebellar pathways. Advanced MRI techniques to assess middle cerebellar peduncles' white matter integrity may be able to strengthen these findings. Follow-up studies using longitudinal models is essential to verify the possibility that the subtle cognitive and psychiatric changes related to cerebellar involvement progress over time to overt and more severe neurological and executive-psychiatric dysfunction as seen in FXTAS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by La Trobe and Monash Universities Human Research Ethics Committees in Melbourne, Australia, and UC Davis Institutional Review Board, USA. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

DH: conception and wrote first draft of manuscript, conducting statistical analysis, interpretation of results, and final review and editing. DL: conception, organization and partial execution of research project, neurological assessments and motor scales scoring, review of statistical analysis, and co-writing the manuscript. PS: conduct and interpretation of neuropsychological and psychiatric pathology assessments and organization and partial execution of research project. FT: conduct and interpretation of genetic molecular assays and review and critique of manuscript. AA: contribution to cognitive testing and scoring, creating study database, and contribution to review and final editing of manuscript. ES: conception and partial execution of research project, neurological assessments and motor scales scoring, and neuropsychological assessments or supervision of assessments. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Grigsby J, Cornish K, Hocking D, Kraan C, Olichney JM, Rivera SM, et al. The cognitive neuropsychological phenotype of carriers of the FMR1 premutation. *J Neurodev Disord.* (2014) 6:28. doi: 10.1186/1866-1955-6-28
2. Hagerman RJ, Hagerman P. Advances in clinical and molecularunderstanding of the FMR1 premutation and fragile X-associated tremor/ataxia syndrome. *Lancet Neurol.* (2013) 12:786–98. doi: 10.1016/S1474-4422(13)70125-X
3. Hagerman RJ, Hagerman P. Fragile X-associated tremor/ataxia syndrome – features, mechanisms and management. *Nat Rev Neurol.* (2016) 12:403–12. doi: 10.1038/nrneurol.2016.82
4. Roberts JE, Tonnsen BL, McCary LM, Ford AL, Golden RN, Bailey DB Jr. Trajectory and predictors of depression and anxiety disorders in mothers with the *FMR1* premutation. *Biol Psychiatry*. (2016) 79:850–7. doi: 10.1016/j.biopsych.2015.07.015
5. Hagerman RJ, Protic D, Rajaratnam A, Salcedo-Arellano MJ, Aydin EY, Schneider A. Fragile X-associated neuropsychiatric disorders (FXAND). *Front Psychiatry.* (2018) 9:564. doi: 10.3389/fpsyt.2018.00564
6. Klusek J, Hong J, Sterling A, Berry-Kravis E, Mailick MR. Inhibition deficits are modulated by age and CGG repeat length in carriers of the FMR1 premutation allele who are mothers of children with fragile X syndrome. *Brain Cogn.* (2020) 139:105511. doi: 10.1016/j.bandc.2019.105511
7. Kraan CM, Hocking DR, Georgiou-Karistianis N, Metcalfe SA, Archibald AD, Fielding J, et al. Impaired response inhibition is associated with self-reported symptoms of depression, anxiety, and ADHD in female FMR1 premutation carriers. *Am J Med Genet B Neuropsychiatr Genet.* (2014) 165B:41–51. doi: 10.1002/ajmg.b.32203
8. Moser C, Schmitt L, Schmidt J, Fairchild A, Klusek J. Response inhibition deficits in women with the FMR1 premutation are associated with age and fall risk. *Brain Cogn.* (2021) 148:105675. doi: 10.1016/j.bandc.2020.105675
9. Shelton AL, Cornish KM, Kraan CM, Lozano R, Bui M, Fielding J. Executive dysfunction in female FMR1 premutation carriers. *Cerebellum.* (2016) 15:565. doi: 10.1007/s12311-016-0782-0
10. Miyake A, Friedman NP, Emerson MJ, Witzki AH, Howerter A, Wager TD. The unity and diversity of executive functions and their contributions to complex "Frontal Lobe" tasks: a latent variable analysis. *Cogn Psychol.* (2000) 41:49–100. doi: 10.1006/cogp.1999.0734
11. Hunter JE, Allen EG, Abramowitz A, Rusin M, Leslie M, Novak G, et al. No evidence for a difference in neuropsychological profile among carriers and noncarriers of the FMR1 premutation in adults under the age of 50. *Am J Hum Genet.* (2008) 83:692–702. doi: 10.1016/j.ajhg.2008.10.021
12. Allen EG, Hunter JE, Rusin M, Juncos J, Novak G, Hamilton D, et al. Neuropsychological findings from older premutation carrier males and their noncarrier siblings from families with fragile x syndrome. *Neuropsychology.* (2011) 25:404–11. doi: 10.1037/a0021879
13. Bourgeois JA, Seritan AL, Casillas EM, Hessl D, Schneider A, Yang Y, et al. Lifetime prevalence of mood and anxiety disorders in fragile X premutation carriers. *J Clin Psychiatry.* (2011) 72:175–82. doi: 10.4088/JCP.09m05407blu
14. Gossett A, Sansone S, Schneider A, Johnston C, Hagerman R, Tassone F, et al. Psychiatric disorders among women with the fragile X premutation without children affected by fragile X syndrome. *Am J Med Genet B Neuropsychiatr Genet.* (2016) 171:1139–47. doi: 10.1002/ajmg.b.32496
15. Loesch DZ, Tassone F, Atkinson A, Stimpson P, Trost N, Pountney DL, et al. Differential progression of motor dysfunction between male and female fragile x premutation carriers reveals novel aspects

of sex-specific neural involvement. *Front Mol Biosci.* (2021) 7:577246. doi: 10.3389/fmolb.2020.577246
16. Kraan CM, Hocking DR, Georgiou-Karistianis N, Metcalfe SA, Archibald AD, Fielding J, et al. Age and CCG-repeat length associated with neuromotor impairments in at-risk females with the FMR1 premutation. *Neurobiol Aging.* (2014) 35:2179.e7-13. doi: 10.1016/j.neurobiolaging.2014.03.018
17. O'Keeffe C, Taboada LP, Feerick N, Gallagher L, Lynch T, Reilly RB. Complexity based measures of postural stability provide novel evidence of functional decline in fragile X premutation carriers. *J Neuroeng Rehabil.* (2019) 16:87. doi: 10.1186/s12984-019-0560-6
18. McKinney WS, Wang Z, Kelly S, Khemani P, Lui S, White SP, et al. Precision sensorimotor control in aging FMR1 gene premutation carriers. *Front Integr Neurosci.* (2019) 13:56. doi: 10.3389/fnint.2019.00056
19. Hocking DR, Kraan CM, Godler DE, Bui QM, Li X, Bradshaw JL, et al. Evidence linking FMR1 mRNA and attentional demands of stepping and postural control in women with the premutation. *Neurobiol Aging.* (2015) 36:1400–8. doi: 10.1016/j.neurobiolaging.2014.11.012
20. O'Keefe, JA, Robertson EE, Ouyang B, Carns D, McAsey A, et al. Cognitive function impacts gait, functional mobility and falls in fragile X-associated tremor/ataxia syndrome. *Gait Posture.* (2018) 66:288–93. doi: 10.1016/j.gaitpost.2018.09.005
21. Kraan CM, Hocking DR, Georgiou-Karistianis N, Metcalfe SA, Archibald AD, Fielding J, et al. Cognitive-motor interference during postural control indicates at-risk cerebellar profiles in females with the FMR1 premutation. *Behav Brain Res.* (2013) 253:329–36. doi: 10.1016/j.bbr.2013.07.033
22. Wang Z, Khemani P, Schmitt LM, Lui S, Mosconi MW. Static and dynamic postural control deficits in aging fragile X mental retardation 1 (FMR1) gene premutation carriers. *J Neurodev Disord.* (2019) 11:2. doi: 10.1186/s11689-018-9261-x
23. Storey E, Bui MQ, Stimpson P, Tassone F, Atkinson A, Loesch DZ. Relationships between motor scores and cognitive functioning in FMR1 female premutation X carriers indicate early involvement of cerebello-cerebral pathways. *Cerebellum Ataxias.* (2021) 8:15. doi: 10.1186/s40673-021-00138-0
24. Loesch D, Huggins R, Hay D, Gedeon A, Mulley J, Sutherland G. Genotype-phenotype relationships in fragile X syndrome: a family study. *Am J Hum Genet.* (1993) 53:1064.
25. Loesch DZ, Hay DA, Mulley J. Transmitting males and carrier females in fragile X–revisited. *Am J Med Genet.* (1994) 51:392–9. doi: 10.1002/ajmg.1320510418
26. Loesch DZ, Huggins R, Petrovic V, Slater H. Expansion of the CGG repeat in fragile X in the FMR1 gene depends on the sex of the offspring. *Am J Hum Genet.* (1995) 57:1408.
27. Postuma R, Lang A, Gagnon J, Pelletier A, Montplaisir J. How does parkinsonism start? Prodromal parkinsonism motor changes in idiopathic REM sleep behaviour disorder. *Brain.* (2012) 135:1860–70. doi: 10.1093/brain/aws093
28. Fitzpatrick LE, Jackson M, Crowe SF. Characterization of cerebellar ataxia in chronic alcoholics using the International Cooperative Ataxia Rating Scale (ICARS). *Alcohol Clin Exp Res.* (2012) 36:1942–51. doi: 10.1111/j.1530-0277.2012.01821.x
29. Harrison AG, Armstrong IT, Harrison LE, Lange RT, Iverson GL. Comparing canadian and american normative scores on the Wechsler Adult Intelligence Scale-Fourth Edition. *Arch Clin Neuropsychol.* (2014) 29:736–46. doi: 10.1093/arclin/acu048
30. Leung JLM, Lee GTH, Lam YH, Chan RCC, Wu JYM. The use of the Digit Span Test in screening for cognitive impairment in acute medical impatients. *Int Psychogeriatr.* (2011) 23:1569–74. doi: 10.1017/S1041610211000792
31. Tombaugh TN. Trail making test A and B: normative data stratified by age and education. *Arch Clin Neuropsychol.* (2004) 19:203–14. doi: 10.1016/S0887-6177(03)00039-8
32. Bayard S, Erkes J, Moroni C. Stroop test: normative data in a sample group of older people and the study of their clinical applications in the assessment of inhibition in Alzheimer's disease. *Arch Clin Neuropsychol.* (2011) 26:653–61. doi: 10.1093/arclin/acr053
33. Kiely KM, Butterworth P, Watson N, Wooden M. The symbol digit modalities test: normative data from a large nationally representative sample of Australians. *Arch Clin Neuropsychol.* (2014) 29:767–75. doi: 10.1093/arclin/acu055
34. Wechsler D. *The Wechsler Adult Intelligence Scale. Administration and Scoring Manual*. 3rd ed. Orlando, FL: The Psychological Corporation (1997).
35. Golden CJ, Freshwater SM. *The Stroop Color and Word Test: Revised Examiner's Manual*. Wood Dale, IL: Stoelting Co. (2002).
36. Reitan RM, Wolfson D. *The Halstead-Reitan Neuropsychological Test Battery*. Tucson: Neuropsychology Press (1985).
37. Smith A. *Symbol Digit Modalities Test*. Los Angeles, CA.: Western Psychological Services (1982).
38. Trouillas P, Takayanagi T, Hallett M, Currier RD, Subramony SH, Wessel K, et al. International Cooperative Ataxia Rating Scale for pharmacological assessment of the cerebellar syndrome. The Ataxia Neuropharmacology Committee of the World Federation of Neurology. *J Neurol Sci.* (1997) 145:205–11. doi: 10.1016/S0022-510X(96)00231-6
39. Fahn S, Elton R, UPDRS Development Committee. The Unified Parkinson's disease rating scale. In: Fahn S, Marsden CD, Calne DB, Goldstein M, editors. *Recent Developments in Parkinson's Disease*. 2nd ed. Florham Park, NJ: Macmillan Healthcare Information (1987). p. 153–63, 293–304.
40. Richards M, Marder K, Cote L, Mayeux R. Interrater reliability of the Unified Parkinson's Disease Rating Scale motor examination. *Mov Disord.* (1994) 9:89–91. doi: 10.1002/mds.870090114
41. Stacy MA, Elble RJ, Ondo WG, Wu SC, Hulihan J, Group TS. Assessment of interrater and intrarater reliability of the Fahn–Tolosa–Marin Tremor Rating Scale in essential tremor. *Mov Disord.* (2007) 22:833–8. doi: 10.1002/mds.21412
42. Storey E, Tuck K, Hester R, Hughes A, Churchyard A. Inter-rater reliability of the International Cooperative Ataxia Rating scale (ICARS). *Mov Disord.* (2004) 19:190–2. doi: 10.1002/mds.10657
43. Derogatis LR. *SCL-90-R Symptom Checklist-90-R Administration, Scoring and Procedures Manual*. Minneapolis, MN: National Computer Systems (1994).
44. Tassone F, Pan R, Amiri K, Taylor AK, Hagerman PJ. A rapid polymerase chain reaction-based screening method for identification of all expanded alleles of the fragile X (FMR1) gene in newborn and high-risk populations. *J Mol Diagn.* (2008) 10:43–9. doi: 10.2353/jmoldx.2008.070073
45. Filipovic-Sadic S, Sah S, Chen L, Krosting J, Sekinger E, Zhang W, et al. A Novel FMR1 PCR method for the routine detection of low abundance expanded alleles and full mutations in fragile X syndrome. *Clin Chem.* (2010) 56:399–408. doi: 10.1373/clinchem.2009.136101
46. O'Halloran CJ, Kinsella GJ, Storey E. The cerebellum and neuropsychological functioning: a critical review. *J Clin Exp Neuropsychol.* (2012) 34:35–56. doi: 10.1080/13803395.2011.614599
47. Friedman NP, Miyake A. Unity and diversity of executive functions: individual differences as a window on cognitive structure. *Cortex.* (2017) 86:186–204. doi: 10.1016/j.cortex.2016.04.023
48. Stuss DT, Alexander MP, Floden D, Binns MA, Levine B, McIntosh AR, et al. Fractionation and localization of distinct frontal lobe processes: evidence from focal lesions in humans. In Stuss DT, Knight RT, editors. *Principles of Frontal Lobe Function*. New York, NY: Oxford University Press (2002). 392–407.
49. Potenza MN, Leung HC, Blumberg HP, Peterson BS, Fulbright RK, Lacadie CM, et al. An fMRI Stroop task study of ventromedial prefrontal cortical function in pathological gamblers. *Am J Psychiatry.* (2003) 160:1990–4. doi: 10.1176/appi.ajp.160.11.1990
50. Watson TC, Becker N, Apps R, Jones MW. Back to front: cerebellar connections and interactions with the prefrontal cortex. *Front Syst Neurosci.* (2014) 8:4. doi: 10.3389/fnsys.2014.00004
51. Battistella G, Niederhauser J, Fornari E, Hippolyte L, Perrin AG, Lesca G, et al. Brain structure in asymptomatic FMR1 premutation carriers at risk for fragile X-associated tremor/ataxia syndrome. *Neurobiol Aging.* (2013) 34:1700–7. doi: 10.1016/j.neurobiolaging.2012.12.001
52. Tassone F, Greco CM, Hunsaker MR, Seritan AL, Berman RF, Gane LW, et al. Neuropathological, clinical and molecular pathology in female fragile X premutation carriers with and without FXTAS. *Genes Brain Behav.* (2012) 11:577–85. doi: 10.1111/j.1601-183X.2012.00779.x
53. Roberts JE, Bailey DB, Mankowski J, Ford A, Sideris J, Weisenfeld LA, et al. Mood and anxiety disorders in females with the FMR1 premutation. *Am J Med Genet B Neuropsychiatr Genet.* (2009) 150:130–39. doi: 10.1002/ajmg.b.30786
54. Seltzer MM, Barker ET, Greenberg JS, Hong J, Coe C, Almeida D. Differential sensitivity to life stress in FMR1 premutation carrier mothers of children

with fragile X syndrome. *Health Psychol.* (2012) 31:612. doi: 10.1037/a0026528

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Allopregnanolone Improves Locomotor Activity and Arousal in the Aged CGG Knock-in Mouse Model of Fragile X-Associated Tremor/Ataxia Syndrome

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Carriers of the fragile X premutation (PM) can develop a variety of early neurological symptoms, including depression, anxiety and cognitive impairment as well as being at risk for developing the late-onset fragile X-associated tremor/ataxia syndrome (FXTAS). The absence of effective treatments for FXTAS underscores the importance of developing efficacious therapies to reduce the neurological symptoms in elderly PM carriers and FXTAS patients. A recent preliminary study reported that weekly infusions of Allopregnanolone (Allop) may improve deficits in executive function, learning and memory in FXTAS patients. Based on this study we examined whether Allop would improve neurological function in the aged CGG knock-in (CGG KI) dutch mouse, B6.129P2(Cg)-Fmr1^{tm2Cgr}/Cgr, that models much of the symptomatology in PM carriers and FXTAS patients. Wild type and CGG KI mice received 10 weekly injections of Allop (10 mg/kg, s.c.), followed by a battery of behavioral tests of motor function, anxiety, and repetitive behavior, and 5-bromo-2′-deoxyuridine (BrdU) labeling to examine adult neurogenesis. The results provided evidence that Allop in CGG KI mice normalized motor performance and reduced thigmotaxis in the open field, normalized repetitive digging behavior in the marble burying test, but did not appear to increase adult neurogenesis in the hippocampus. Considered together, these results support further examination of Allop as a therapeutic strategy in patients with FXTAS.

Keywords: : FXTAS, allopregnanolone, mouse, behavior, adult neurogenesis

INTRODUCTION

Approximately 1.5 million individuals in the US are carriers of the Fragile X premutation (PM), with prevalence estimates ranging from 1:209 to 1:250 in females and 1:430 to 1:800 in males (Hunter et al., 2008; Hantash et al., 2011; Tassone et al., 2012). Many carriers develop neurological and psychological problems over their lifespan including anxiety, depression, and poor motor

performance (Aziz et al., 2003; Moore et al., 2004; Cornish et al., 2005; Farzin et al., 2006; Coffey et al., 2008; Koldewyn et al., 2008; Bourgeois et al., 2011; Goodrich-Hunsaker et al., 2011). Carriers of PM are also at risk for developing Fragile X-associated tremor/ataxia syndrome (FXTAS), a late onset neurodegenerative disorder characterized by tremors, ataxia, brain atrophy and cognitive decline (Hagerman et al., 2001). The chances of developing FXTAS increase dramatically with age, with approximately 40% of males and 8–11% of female PM carriers over the age of 50 developing FXTAS (Rodriguez-Revenga et al., 2009). Indeed, FXTAS may be one of the more common causes of tremor/ataxia in older adults (Jacquemont et al., 2004). Because of the dramatic increase in the number of individuals reaching the age of 65, and increasing life expectancy, the numbers of FXTAS patients will increase accordingly, further highlighting the importance of research on PM and FXTAS (Jacquemont et al., 2004). At the present time there are no effective treatments to improve neurological function and overall quality of life for many affected PM carriers and FXTAS patients (Hagerman et al., 2008, 2009, 2010; Berry-Kravis et al., 2011). Therefore, it is extremely important to understand the underlying pathology in FXTAS, establish its developmental time course, and develop rational treatment strategies.

The goal of this preclinical study was to determine whether chronic treatment with the neurosteroid Allopregnanolone (Allop, 3α-hydroxy-5α-pregnan-20-one) would improve motor function and anxiety-like behaviors in a knock-in mouse model (i.e., CGG_{ex} KI) of the PM and FXTAS (Willemsen et al., 2003; Van Dam et al., 2005; Berman and Willemsen, 2009). This mouse KI model recapitulates much of the pathology in the PM and FXTAS (Berman and Willemsen, 2009). This includes a 2–3-fold elevation in *Fmr1* mRNA in brain and the accumulation over time of ubiquitin-positive intranuclear inclusions in both neurons and astrocytes. The KI model also exhibits motor deficits on the rotarod and ladder walking tasks (Van Dam et al., 2005; Hunsaker et al., 2011), cognitive impairments in several spatial memory tasks (Van Dam et al., 2005; Hunsaker et al., 2009), and increased anxiety in the elevated plus-maze (Berman and Willemsen, 2009), modeling clinical symptoms often seen in PM carriers and in patients with FXTAS.

Allop is a neurosteroid that enhances $GABA_A$ function, stimulates neurogenesis and has been shown to improve cognitive function in animal models of neurodegenerative disease including Alzheimer's disease (Brinton and Wang, 2006a,b; Wang et al., 2010). Allop has also has been found to enhance adult neurogenesis in the hippocampal subgranular zone (SGZ) (Genazzani et al., 1998; Kim et al., 2006; Herd et al., 2007; Nilsen et al., 2007; Wang et al., 2010; Chen et al., 2011; Noorbakhsh et al., 2011; Turkmen et al., 2011; Holden et al., 2012; Singh et al., 2012; Sun et al., 2012). Allop appears to stimulate neurogenesis in the hippocampus through a $GABA_A$ mediated increase in intracellular calcium, leading to increased expression of genes for proteins that promote cell proliferation (Wang et al., 2005, 2008). This is relevant because GABAergic transmission appears to be abnormal in CGG_{ex} KI mice. D'Hulst et al. (2009) reported that $GABA_A$ receptor subunits, transporters (GAT 1 and 2), and GAD are upregulated in the cerebellum of CGG_{ex} KI mice, suggesting a compensatory response to decreased GABAergic transmission. Levels of the GABA vesicular transporter VGAT are also lower in hippocampal neurons cultured from CGG_{ex} KI mice suggesting reduced presynaptic levels of GABA (Cao et al., 2012). In addition, reduced GABAergic signaling in cultured neurons from CGG_{ex} KI mice results in burst-firing of neurons and abnormal patterns of Ca^{+2} oscillations that can be rescued by culturing neurons in the presence of Allop (Cao et al., 2012). Therefore, in this study we examined the ability of Allop treatment to ameliorate behavioral pathology in a mouse model of the PM and FXTAS.

A recent open-label study assessed whether Allop given as 12 weekly infusions (2–6 mg) to 6 FXTAS patients would improve clinical symptoms, brain electrophysiological activity, and MRI measurements of brain deterioration and white matter pathology (Wang et al., 2017). Treatment improved executive functioning, episodic memory and learning, and increased N400 repetition effect amplitude as an index of brain activity. Overall deterioration of the brain on MRI was not significantly affected, although some patients showed improvement. In a separate report in 2019, plasma pharmacometabolomics and lymphocytic mitochondria function were assessed at baseline and within 48 h from the last infusion, and Allop treatment altered GABA metabolism and reduced markers of oxidative stress (Napoli et al., 2019). Considered together, results of this limited pilot clinical research project indicate that Allop may improve psychological and cognitive performance in FXTAS, and that further research is warranted, including preclinical studies such as the present study.

MATERIALS AND METHODS

Animals

Twenty-three male B6.129P2(Cg)-Fmr1^{tm2Cgr} "dutch CGG KI mice" (CGG KI) aged 13–16 months old, and 18 aged-matched wild type (WT) littermates were derived from a total of 18 litters bred at the UC Davis Neurotherapeutics Institute. The range of CGG trinucleotide repeat expansions for CGG KI mice was 67–197 (Mean 112 ± 10.5), and 10.0 ± 0.4 for WT mice. The mice were randomly assigned to receive 10 once-weekly injections of 10 mg/kg, s.c. Allopregnanalone (Allop) or β-cyclodextrin vehicle control (Veh) in one of 4 treatment groups: wild type-vehicle (WT-Veh; n = 9), wild type-Allop (WT-Allop; n = 9), CGG KI-vehicle (CGG KI-Veh; n = 11), CGG KI-Allop (CGG KI-Allop; n = 12). This drug injection protocol was patterned after that reported to improve cognitive performance in a mouse model of Alzheimer's disease (Singh et al., 2012), and the Allop treatment paradigm was designed to avoid the potential cognitive impairments observed with acute exposure (Johansson et al., 2002, 2016). In order to avoid any transient effects of Allop as an acute $GABA_A$ receptor modulator, mice were given a 1-week washout period after the last Allop injection before being evaluated across a series of behavioral tasks to assess motor coordination, balance, locomotor activity, and arousal as outlined in **Figure 1**. All experiments were conducted during the light phase of the light/dark cycle and were pre-approved by the University of California, Davis IACUC and in accordance with

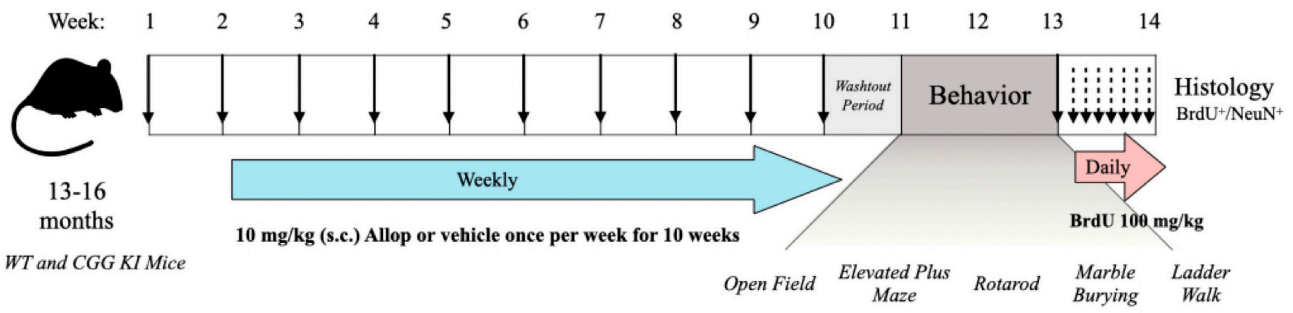

FIGURE 1 | Timeline for the experimental procedures. WT and CCG KI mice between the ages of 13–16 months old were treated once weekly with allopregnanolone (Allop) (10 mg/kg s.c.) or β-cyclodextrin vehicle control. Following the last injection, mice were left undisturbed for a 1-week drug washout period and then assessed for behavioral differences across a battery of motor and arousal tasks. Then, mice were injected once daily with 5-bromo-2′-deoxyuridine (BrdU, 100 mg/kg i.p.) for seven consecutive days and their brains were then processed for evidence of neurogenesis.

the policies of the National Institutes of Health for the humane care and use of laboratory animals.

Genotyping

CGG repeat length was determined using methods previously described by Hunsaker et al. (2011). Briefly, DNA was extracted from mouse tails by incubation with 10 mg/mL Proteinase K (Roche Diagnostics; Mannheim, Germany) in 300 μL lysis buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, 150 mM NaCl, 1% SDS overnight at 55°C. One hundred μL saturated NaCl was then added and the suspension was centrifuged. One volume of 100% ethanol was added, gently mixed, and the DNA was pelleted by centrifugation and the supernatant discarded. The DNA was washed and centrifuged in 500 μL 70% ethanol and then dissolved in 100 μL milliQ-H_2O. CGG repeat lengths were determined by PCR using the Expanded High Fidelity Plus PCR System (Roche Diagnostics). Approximately 500–700 ng of DNA was added to 50 μL of PCR mixture containing 2.0 μM/L of each primer, 250 μM/L of each dNTP (Invitrogen; Tigard, OR), 2% dimethyl sulfoxide (Sigma-Aldrich; St. Louis, MO), 2.5 M Betaine (Sigma-Aldrich), 5 U Expand HF buffer with Mg (7.5 μM/L). The forward primer was 5′-GCTCAGCTCCGTTTCGGTTTCACTTCCGGT-3′ and the reverse primer was 5′-AGCCCCGCACTTCCACCACCA GCTCCTCCA-3′. PCR steps were 10 min denaturation at 95°C, followed by 34 cycles of 1 min denaturation at 95°C, annealing for 1 min at 65°C, and elongation for 5 min at 75°C to end each cycle. PCR ended with a final elongation step of 10 min at 75°C. DNA CGG band sizes were determined by running DNA samples on a 2.5% agarose gel and staining DNA with ethidium bromide.

Open Field

Locomotor behaviors were tested by placing mice in an empty Plexiglas arena (30 cm × 30 cm × 38 cm) and allowing them to freely explore the environment for 5 min. The total time spent in the center and margin of the arena as well as the number of line crosses in a 3 × 3 grid were counted by researchers blind to genotype and treatment conditions.

Marble Burying

Mice were habituated for 10 min to clean Plexiglas cages (37 cm × 14 cm × 12.5 cm) filled with a 4 cm thick layer of clean corncob bedding for 10 min. Following habituation, animals were returned to their home cage and 15 glass marbles were laid out in five rows of three marbles placed equidistance apart. Mice were then returned to the cages and allowed to explore under dim illumination for 10 min. At the end of the 10 min period, animals were gently removed from the testing cages and the number of marbles buried was recorded. Only marbles covered by 75% or more bedding were counted as buried.

Rotarod

Balance and motor coordination were assessed using a Rotamex-5 rotarod with infrared photocell detection (Columbus Instr., Columbus, OH). Mice were first given an initial training session by placing them on the rotarod rotating at a constant speed of 4 RPM for 120 sec. Mice that fell were immediately reloaded and allowed to complete the training session. The following day, mice were placed on the rotarod at an initial speed of 4 RPM that accelerated by 1.0 RPM every 10 s. A trial was terminated when a mouse fell from the rod at which time the latency to fall was recorded. Each mouse was tested twice. Mean performance time was defined as the average time the mouse remained on the rotarod across trials.

Elevated Plus Maze

Anxiety in the mice was assessed using the elevated plus maze (Handley and Mithani, 1984; Walf and Frye, 2007). The Plexiglas apparatus consisted of two open arms (30 cm × 5 cm × 0.5 cm) and two perpendicular closed arms (30 cm × 5 cm × 15 cm) extending from a central platform. The entire maze was elevated approximately 1 m from the floor. Mice were placed in the central platform and allowed to freely explore the maze for 5 min. The total number of entries in the open arm, as defined by all four paws outside the central zone, as well as the total time spent in the open and closed arms were recorded. Percent of open arm exploration time was calculated as the time in the open arm divided by the total time in both the open and closed arms.

Ladder Walk

The ladder walk task was conducted as previously described (Hunsaker et al., 2011). The apparatus consisted of two, 28 cm tall × 65 cm long black walls separated by 10 cm. The floor was elevated 10 cm from the bottom of the walls and was made from 43 parallel 1 mm diameter bars separated by 1.5 cm. Mice were placed in the apparatus and allowed to freely explore the apparatus for 2 min while being video recorded. The digital recordings were scored for the number of times a foot (forelimb or hindlimb) slipped through the floor bars as previously described (Hunsaker et al., 2011). The digital recordings were scored independently by two experimenters blinded to the genotype and treatment of the animals.

Histology

Immediately following the final behavioral task, mice were injected once daily with 5-bromo-2′-deoxyuridine (BrdU) (100 mg/kg, i.p.) for seven consecutive days and then sacrificed to quantify hippocampal neurogenesis as previously described (Wojtowicz and Kee, 2006). Briefly, brain tissue was fixed with 4% paraformaldehyde, flash frozen, and serial 40 μm sections were cut anterior-posterior through the hippocampus. Sections were mounted on glass slides, rinsed 3 times for 10 min each with 0.1 M phosphate buffered saline (PBS) followed by 0.5% hydrogen peroxide in methanol for 15 min. Following another series of rinses, sections were submerged in 2N HCL solution for 20 min at 37 degrees Celsius, rinsed 3 times for 10 min each, and placed in blocking solution of 3% BSA, 3% goat serum, and 0.3% triton X for 1 h. Sections were then incubated for 18 h in blocking solution containing 1:50 anti-BrdU antibody (Accurate Chemical) and 1:100 anti-NeuN (EMD Millipore). The following day, sections were rinsed 6 times for 5 min each with 0.1 M PBS containing 0.3% Triton X and then incubated in blocking solution containing fluorescent-labeled secondary antibodies for 2 h. Sections were counter stained in a 1:500 solution of DAPI, rinsed 6 times for 5 min. each in 0.1 M PBS, and coverslipped with fluoromount G. The number of $BrdU^+$ and double labeled $BrdU^+/NeuN^+$ cells were quantified in the subgranular zone of the dentate gyrus, defined as a narrow band 1–2 cells thick between the granule cell layer and the hilus (Kempermann et al., 1997), on every fifth section. Co-localization was determined by focusing through each section and toggling between filter sets to visually establish that immunofluorescence for BrdU and NeuN was from the same cell.

Statistical Analysis

Data were analyzed using R version 4.0.4 (2021) using the "stats" and "pscl" packages. Mortality measures (i.e., number of deceased mice throughout the study) were evaluated across genotype and treatment conditions using chi-square test for independence. For behavioral measures, assumptions of normality and linearity were evaluated using the Shapiro-Wilks test and assessed visually using qqplots and histograms. Count data (number of open arm entries, marble burying, and ladder walk slips, and cell counts) containing excess numbers of zeros were assessed using Zero-Inflated Poisson regression followed by *post hoc* planned comparisons. Similarly for continuous measures (open field, rotarod), data were analyzed using Wilcoxon-Mann-Whitney planned comparisons to determine if (1) wild type mice treated with vehicle (WT-Veh) differed significantly from vehicle-treated CGG KI mice (CGG KI-Veh); (2) CGG KI mice treated with Allop (CGG KI-Allop) differed significantly from CGG KI-Veh mice; and (3) if wild type mice treated with Allop (WT-Allop) differed significantly from WT-Veh. All analyses were two-tailed, and values of $p \leq 0.05$ were considered statistically significant. All graphs show sample medians and inter-quartile range (IQR).

RESULTS

Mortality and Body Weight

A total of 4 CGG KI mice and 2 wild type mice died during the time-course of the study. Of those deceased, 1 CGG KI vehicle-injected and 2 wild type Allop-injected mice were euthanized before the end of the injection series due to skin lesions at the site of the subcutaneous injections (**Table 1**). Chi-square analysis confirmed that there were no differences in the number of mice that died or were euthanized between genotype [X^2 (1, $N = 41$) = 0.81, $p = 0.37$] or drug treatment, [X^2 (1, $N = 41$) = 0.04, $p = 0.84$]. There were no significant differences in body weight among groups before or after the last injection of either saline or Allop, and no significant change in body weight between the 1st and 10th injection due to treatment or genotype.

Locomotor Activity and Arousal

Locomotor and anxiety-related behaviors for CGG KI and wild type mice were measured in the open field and elevated plus maze tasks (**Figures 2A–D**). Overall activity in the open field was quantified by the number of line crosses over a 5 min period. As shown in **Figure 2A**, CGG KI-Veh mice had significantly fewer line crosses (i.e., lower activity) compared to WT-Veh mice, and this effect was ameliorated in the CGG KI mice by Allop treatment. Statistical analysis of locomotor activity in the open field, measured by the number of line crosses, confirmed that activity was significantly lower in CGG-Veh mice compared to WT-Veh mice ($p = 0.018$). Allop treatment did not significantly affect Wild type mice (i.e., WT-Veh vs. WT-Allop, $p = 0.790$), but significantly increased activity in the CGG KI mice (CGG KI-Veh vs. CGG KI-Allop, $p = 0.007$). In fact, Allop treatment restored locomotor activity in the CGG KI mouse to a level similar to vehicle-treated wild type mice.

As shown in **Figure 2B**, there was a significant effect of Allop treatment on time spent in the margin for CGG KI mice.

TABLE 1 | Sample sizes for each genotype and treatment group.

Genotype	Treatment	Alive	Deceased
Wild type	Vehicle	9	0
Wild type	Allop	7	2
CGG repeat	Vehicle	9	2
CGG repeat	Allop	10	2

There were no significant differences in the number of mice that died or were euthanized between groups.

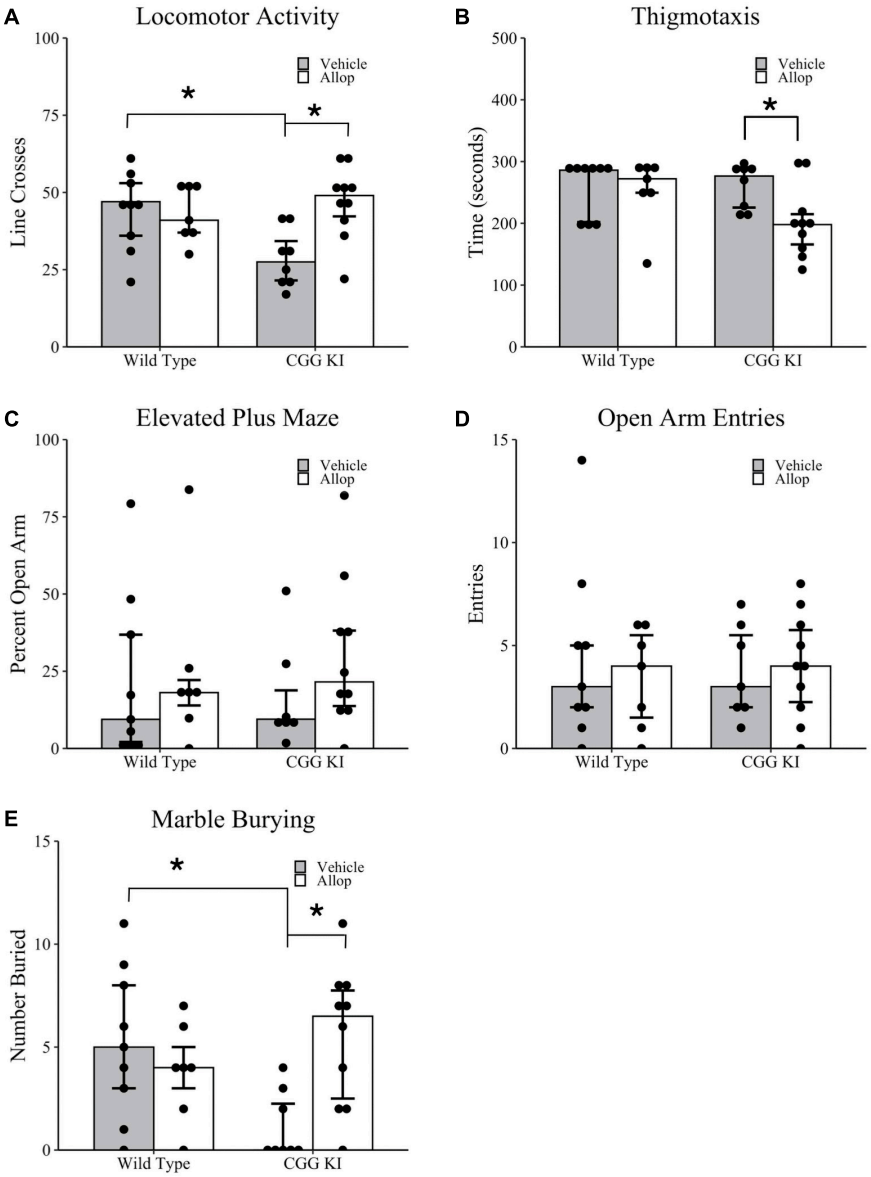

FIGURE 2 | Locomotor activity, arousal, and anxiety-associated behaviors in the CGG KI and wild type mice. Mice were treated for 10 weeks with Allop or vehicle control and evaluated for locomotor activity and thigmotaxis in the open field task **(A,B)**, anxiety-like measures in the elevated plus maze **(C,D)**, and repetitive digging behavior in the marble burying task **(E)**. Reported are **(A)** the number of lines crossed during a 5-min open field exploration; **(B)** the total time spent exploring the margins of the open field arena (i.e., thigmotaxis); **(C)** the percent time exploring the open arms in the elevated plus maze, as well as **(D)** the total number of entries into the open arms; **(E)** the percent of marbles buried in the marble burying task. *$p < 0.05$ as determined by Wilcoxon-Mann-Whitney (Locomotor Activity and Thigmotaxis) or Zero-Inflated Poisson Regression (Elevated Plus Maze and Marble Burying) followed by pairwise planned comparisons. Plots represent individual mice; bars represent median ± IQR. Wild type-vehicle (n = 9), wild type-Allop (n = 7), CGG KI-vehicle (n = 8), CGG KI-Allop (n = 10).

Planned comparisons showed that WT-Veh mice did not differ significantly in margin time from either WT-Allop (p = 0.831) or CGG KI-Veh (p = 0.699). However, CGG KI-Allop treated mice spent less time in the margin compared to CGG KI-Veh (p = 0.050).

In the elevated plus maze, there was no difference in percent time in the open arms between vehicle treated mice of both genotypes (p = 0.918) and no effect of treatment on CGG KI-Allop (p = 0.133) or WT-Veh (p = 0.491). On average, mice only spent approximately 24% of their exploration time in the open arms of the maze (**Figure 2C**). Importantly, there were no differences in the total number of open arm entries between genotypes (z = 0.04, p = 0.631) or treatment (z = 0.02, p = 0.851), suggesting that motor activity did not impact elevated plus maze performance (**Figure 2D**).

Figure 2E shows the results of Allop treatment on marble burying used as a test of repetitive digging behavior (Thomas et al., 2009). There was a significant treatment by genotype

interaction (z = 2.26, p = 0.024), in the number of marbles buried in a 10-min marble burying task. Planned comparisons confirmed that CGG KI-Veh mice buried fewer marbles than WT-Veh and CGG KI-Allop mice ($p < 0.001$ for both comparisons).

Motor Coordination

Motor coordination was evaluated in the rotarod (**Figure 3A**) and ladder walk tasks (**Figure 3B**). In the rotarod task, there were no differences in total time on the rotarod between genotypes, WT-Veh compared to CGG KI-Veh (p = 0.630), and no differences between treatment conditions, CGG KI-Veh compared to CGG KI-Allop (p = 0.897) and WT-Veh compared to WT-Allop (p = 0.536). On the ladder walk test, a poisson regression revealed a marginally significant effect of treatment (z = 1.93, p = 0.053) based on genotype (z = 2.08, p = 0.038). Specifically, Allop treatment in WT mice reduced the number of foot slips compared to WT-Veh controls (p = 0.051), but no differences were observed between Allop and Vehicle treatment in CGG KI mice (p = 0.425) (**Figure 3B**).

Dentate Gyrus Neurogenesis

The effects of 10 weeks of once weekly Allop treatment on cell proliferation and neurogenesis in the hippocampus are shown in **Figure 4**. Images of the dentate gyrus labeled with BrdU (green fluorescence, **Figure 4A**) and NeuN (red fluorescence, **Figure 4B**) were quantified for the number of colocalized $BrdU^+/NeuN^+$ cells (yellow fluorescent cells; see insert, **Figure 4C**). Poisson regression analysis for the total number of $BrdU^+$ cells confirmed a significant treatment effect of Allop ($z = -8.42$, $p < 0.001$) but not for genotype ($z = -1.42$, p = 0.157). **Figure 4D** shows a greater number of $BrdU^+$ cells in both WT-Allop (p = 0.003) and CGG KI-Allop ($p < 0.001$) compared to genotype-matched vehicle controls. There was no significant effect of genotype on the total number of $BrdU^+/NeuN^+$ double-labeled cells in the dentate gyrus (**Figure 4E**), classified as adult proliferating neurons (i.e., adult neurogenesis), and the effect of AlloP treatment on $BrdU^+/NeuN^+$ double-labeled cells did not reach statistical significance ($z = -1.67$, p = 0.095). However, there was a significant treatment by genotype interaction (z = 2.46, p = 0.014). Planned comparisons confirmed a small increase in the number of double-labeled $BrdU^+/NeuN^+$ cells in CGG KI-Allop mice compared to genotype-matched CGG KI-Veh mice (p = 0.024). **Figure 4F** shows the total number of cells that labeled for BrdU, but not NeuN ($BrdU^+/NeuN^-$), calculated as the difference between total $BrdU^+$ cells labeled and $BrdU^+/NeuN^+$ double labeled cells. This population of BrdU labeled cells likely includes proliferating astrocytes, oligodendrocytes and microglia, as well as young neurons not expressing NeuN although they were not further classified in this study. Interestingly, there was a significant effect of treatment on the number of non-neuronal $BrdU^+/NeuN^-$ cells ($z = -8.57$, $p < 0.001$). Planned group comparisons showed the number of $BrdU^+/NeuN^-$ cells was significantly larger in the CGG KI-Allop mice compared to the CGG KI-Veh mice ($p < 0.001$), and in the WT-Allop mice compared to genotype-matched WT-Veh controls ($p < 0.001$). No effect of genotype were observed between WT-Veh mice and CGG KI-Veh groups (p = 0.923).

DISCUSSION

The CGG KI mouse is a well-validated and established pre-clinical tool for modeling the hallmark behavioral and neurobiological features of the Fragile X PM and FXTAS. This includes motor dysfunction, ubiquitin-positive inclusions, and disruptions in GABAergic signaling (Brouwer et al., 2008; D'Hulst et al., 2009; Berman et al., 2014). While treatments for FXTAS are, to date, only palliative, it has been hypothesized that pharmacological agents that target the GABAergic system may improve neurological function or delay disease progression. In particular, Allop has undergone an initial open-label clinical trial to test its efficacy in reversing the motor and cognitive deficits in a small population of elderly men with FXTAS (Wang et al., 2017; Napoli et al., 2019). Given the high degree of construct validity observed in the CGG KI mouse, we tested whether a similar regimen of Allop in aged CGG KI mice would show efficacy in reducing the behavioral pathologies observed in this mutant mouse model. Our results demonstrated significant improvements in locomotor activity in the open field, but without concomitant improvements in coordination and balance in the rotarod or ladder walk tests. Together, these data provide additional evidence supporting a potential therapeutic effect of Allop on specific FXTAS-associated behavioral deficits.

In humans, premutation carriers show longer, slower motor movements and reaction times that worsen with age (Shickman et al., 2018). Our aged CGG KI mice showed similar deficits in locomotor activity and arousal given the observed reductions in line crosses in the open field task and reduced marble burying activity. The marble burying task in particular revealed a near absence of buried marbles in the CGG KI-Veh mice, likely due to deficits in motor activity and general arousal as a result of the insertion of the CGG-repeat. Treatment with Allop for 10 weeks restored these deficits in motor activity and arousal in both the marble burying and open field tasks to levels observed in wild type controls. These convergent phenotypes across multiple behavioral assays (i.e., open field and marble burying) serve as a measure of reliability in Allop's restorative effects on motor activity. Interestingly, 10 weeks of Allop treatment did not further enhance arousal levels in wild type mice, underscoring the specificity of targeting GABAergic systems to restore, but not enhance, locomotor activity. While these improvements in motor activity would suggest a therapeutic potential for Allop, similar restorative responses were not observed in measures of anxiety, namely the elevated plus maze and thigmotaxis (time in center) measures in the open field arena. Performance on the elevated plus maze task declines with age in the C57BL/6 mouse strain, with aged-mice exhibiting fewer entries and reduced time in open-arm exploration (Sukoff Rizzo et al., 2018; Shoji and Miyakawa, 2019). Similar to our observations in the rotarod and ladder rung task, our observed similarities in elevated plus-maze performance between aged wild type and CGG KI mice would suggest that natural age-related declines

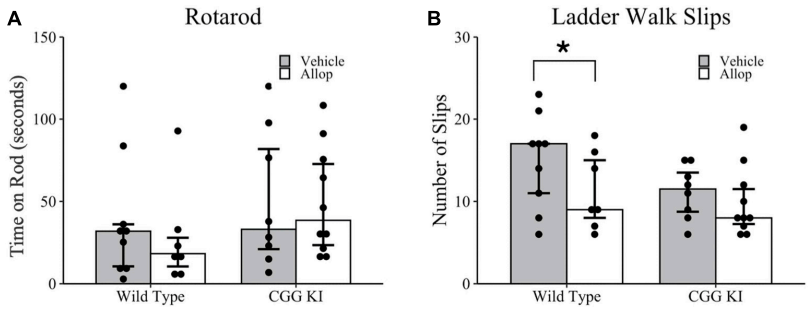

FIGURE 3 | Behavioral assessments of balance and motor coordination in response to Allop in the wild type and CGG KI mouse. Following 10 weeks of Allop treatment mice were evaluated for changes in balance on the rotarod task and motor coordination in the ladder rung task. **(A)** No differences were observed in the average time to fall in the rotarod task. **(B)** In the ladder rung task, mice were evaluated for total number of slips. *$p < 0.05$ as deteremined by Wilcoxon-Mann-Whitney (Rotarod) or Poisson Regression (Ladder Walk) followed by pairwise planned comparisons. Plots represent individual mice; bars represent median ± IQR. Wild type-vehicle ($n = 9$), wild type-Allop ($n = 7$), CGG KI-vehicle ($n = 8$), CGG KI-Allop ($n = 10$).

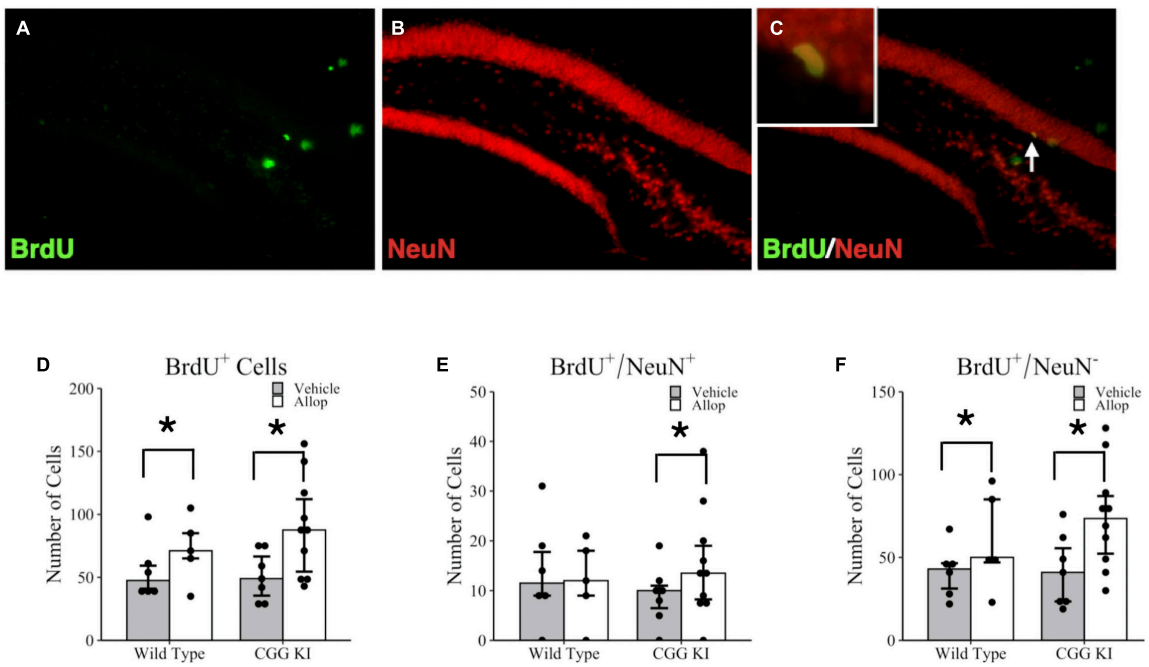

FIGURE 4 | Hippocampal cell proliferation and adult neurogenesis in the brains of CGG KI and wild type mice. Fluorescent photomicrographs of **(A)** $BrdU^+$ cells (green fluorescence) in the subgranular zone of the hippocampus. **(B)** Neurons were labeled with NeuN (red fluorescence), and **(C)** newly generated neurons are co-labled $BrdU^+/NeuN^+$ and appear yellow (high magnification insert). Images shown at 20x magnification. **(D)** The total number of $BrdU^+$ cells in the subgranular zone of WT and CGG KI mice treated with Allop or vehicle for 10 weeks. **(E)** The total number of double labeled $BrdU^+/NeuN^+$ cells reflecting adult neurogenesis in the dentate gyrus of the hippocampus. **(F)** The number of new cells not labeled with NeuN ($BrdU^+/NeuN^-$) was calculated as the total number of number of $BrdU^+$ cells in **(D)** minus the total number of $BrdU^+/NeuN^+$ in **(E)**. *$p < 0.05$ as determined by poisson regression ($BrdU^+$ and $BrdU^+/NeuN^-$) or zero-inflated poisson regression (double-labeled $BrdU^+/NeuN^+$) analysis followed by pairwise planned comparisons. Plots represent aggregated count data of individual mice; bars represent median + IQR. Wild type-vehicle ($n = 5$), wild type-Allop ($n = 6$), CGG KI-vehicle ($n = 7$), CGG KI-Allop ($n = 10$). BrdU, 5-bromo-2′-deoxyuridine.

in performance may be masking earlier anxiety-like effects of the CGG premutation reported in younger CGG KI mice. Therefore, the potential therapeutic effects of Allop treatment on anxiety-like measures remain inconclusive given our inability to effectively evaluate premutation-induced changes in elevated plus-maze performance.

Motor coordination, ataxia and balance are among the hallmark symptoms of FXTAS, and the CGG KI mouse is characterized to show similar deficits in movement and coordination including increases in foot slips in the ladder rung task (Hunsaker et al., 2011) and changes in rotarod performance (Van Dam et al., 2005). Unexpectedly, we did not observe these differences in motor function between our aged wild type and CGG KI mice in either test, regardless of Allop treatment. These results are similar to the study by Van Dam et al. (2005) who also found that rotarod performance

did not differ significantly between 20-, 52-, and 72-week-old CGG KI and control mice. Only when genotypes were analyzed separately did they observe evidence of a significant age-related decline in rotarod performance in 52- and 72-week-old CGG mice compared to 20-week-old genotype-matched mice. While procedural differences between the present study and that of Van Dam et al. (2005) make it difficult to compare studies, such as rotarod apparatus (i.e., Rotamex 5 vs. Ugobasile), pretesting adaptation trials (i.e., a single vs. 2 min trials) and time between adaptation and testing (i.e., 24 h vs. immediate), it is clear that aged CGG KI and control mice did not differ significantly in either study. Previous studies have used a combination of young and old-aged mice (4–16 months) (Hunsaker et al., 2011; Wenzel et al., 2019), but the behavioral phenotype of CGG KI mice at advanced ages, such as the mice used in the present study, has not been well characterized. This is important given that C57BL/6 mice exhibit age-related changes in behaviors including increased anxiety and decreases in motor performance (Sukoff Rizzo et al., 2018; Shoji and Miyakawa, 2019). As a result, natural age-related declines in wild type mouse motor performance (e.g., rotarod and ladder rung test) may equal declines in the aged CGG KI mice, thereby masking differences between wild type and KI mice previously reported in younger mice. Moreover, Allop did not alter rotarod motor performance in either wild type or CGG KI groups, suggesting that weekly treatment with Allop does not improve motor function when started at an advanced age. In a recent pilot study of 6 individuals with FXTAS, 12-weeks Allop did not improve tremor or ataxia (Napoli et al., 2019) corroborating our preclinical findings in the CGG KI mouse. As a result, our data would suggest that targeting GABAergic function with weekly Allop treatment is not sufficient to restore motor coordination deficits resulting from age-related declines in motor function. However, it remains unexplored whether treatment at an earlier age could mitigate or delay the onset of pathology in premutation carriers before the full onset of FXTAS symptomatology.

Allop is an active metabolite of the naturally occurring neurosteroid progesterone with numerous actions in the central nervous system. Allop and other neurosteroids exert numerous classic and non-classic actions in the central nervous system, including promoting genomic and non-genomic cell-cycle functions modulating cell proliferation, cell shape, and gene expression (Garcia-Segura et al., 1996, 1999; Jordan, 1999; Nichols, 1999). Previous studies have demonstrated Allop's ability to promote adult glial cell health and proliferation in addition to its effects on neurogenesis (for review see Faroni and Magnaghi, 2011). Allop is also an allosteric modifier of the $GABA_A$ receptor and can stimulate neurogenesis in the hippocampus (Baulieu et al., 2001). Given the known proliferative effects of Allop on hippocampal neurogenesis both *in vitro* (Wang et al., 2005) and *in vivo* (Wang et al., 2010), we examined whether the locomotor and arousal improvements we observed in our CGG KI mice were accompanied by concomitant increases in adult neurogenesis in the dentate gyrus. Adult hippocampal neurogenesis has been demonstrated in all mammalian species studied to date including mouse (Kempermann et al., 1997; Zhao et al., 2006), rat (Kuhn et al., 1996), non-human primate (Gould et al., 1999) and human (Eriksson et al., 1998). The function of adult neurogenesis is still unclear, but a growing body of evidence indicates its importance in processing of sensory stimuli such as spatial cues necessary for behavioral discrimination (Tuncdemir et al., 2019). Ten weeks of treatment with Allop did not provide compelling evidence of increased neurogenesis in either aged wild type or CGG KI mice. However, this observation is tempered by the fact that in the present study group sizes were relatively small, and numbers of proliferating neurons in the dentate gyrus were also comparatively small, possibly due to the advanced age of the mice used in this study. Adult neurogenesis in the hippocampus declines with age in mice (Kempermann et al., 2002; Yang et al., 2015) and rats (Kuhn et al., 1996), although stable levels of human hippocampal adult neurogenesis have been reported throughout aging (Boldrini et al., 2018). Therefore, the potential for Allop to stimulate adult neurogenesis in the aged rodent hippocampus may be somewhat limited, making it more difficult to document potential drug effects on neurogenesis. It is also possible that the dosage or timing of injections were insufficient to stimulate neuronal proliferation in aged mice.

Ten weeks of Allop injections did significantly increase the total numbers of BrdU-labeled cells that were not $NeuN^+$ in both wild type and CGG KI dentate gyrus. The identity of these proliferating cells was not further examined in the present study, and will need to be determined in future studies. One plausible explanation for this low level of $BrdU^+/NeuN^+$ double labeling may be a result of the timing of BrdU injections and time of sacrifice before histological evaluation. Specifically, Snyder et al. (2009) found that newly generated neurons do not express NeuN until 2–3-weeks after birth, a time period longer than used in the present study. Although we adopted a substantially different BrdU injection regimen to Snyder et al. (2009), their results open the possibility that many of the $BrdU^+$ only cells in our AlloP-exposed mice may include immature neurons not yet expressing the NeuN neuronal marker (Snyder et al., 2009). If so, it is possible that the increase in $BrdU^+$ cells (**Figure 4D**) included new neurons stimulated by AlloP treatment but not yet expressing NeuN. Alternatively, total number of $BrdU^+$ cells in 4D includes astrocytes, oligodendrocyte precursor cells, oligodendrocytes and microglia. Gliogenesis in the adult mammalian hippocampus is well documented (Rietze et al., 2000; Wang et al., 2005; Nixon et al., 2008), and in a triple transgenic mouse model of Alzheimer's disease (3xTgAD), chronic Allop treatment promoted not only neurogenesis, but oligodendrogenesis (Irwin and Brinton, 2014; Chen et al., 2020). It is notable that this increased proliferation of $BrdU^+$ cells by Allop treatment was observed across wild type and CGG KI hippocampus, although planned comparisons indicated that the effect was mainly due to the drug's effects in the CGG KI mouse hippocampus. Glia constitute roughly half of the cells of the central nervous system and provide important functions including homeostasis, neural development, migration, synaptogenesis, synaptic transmission and neural plasticity (Allen and Lyons, 2018). In view of the importance of glia for CNS function, and the possible occurrence

of non-cell autonomous pathology from glial to neurons in FXTAS (Wenzel et al., 2019), it is possible that proliferation of one or more glial subtypes may have contributed to the behavioral results of the present study and this should be examined in future studies.

Considered together, our data provide evidence that 10 weeks of Allop treatment in aged-CGG KI mice improves deficits in locomotor activity and repetitive digging behavior in the marble burying test. Several behavioral measures evaluated in these aged mice, including rotarod and ladder walk, did not show differences between CGG KI and wild type controls in contrast to what has been reported in young CGG KI mice (Berman et al., 2014). This prevents the drawing of any conclusions about possible Allop effects on these types of behaviors. One possibility is that age-related declines in motor performance in very old wild type C57 mice may reach the same level as that of CGG KI mice, making it difficult to compare and identify impaired motor performance in aged CGG KI mice. Given the myriad effects of Allop in modulating both cellular and neurochemical systems in the central nervous system, it is important in the future to examine whether treatment with Allop in earlier stages of the PM pathology may be more effective in delaying the onset of the behavioral and histopathological deficits induced by the CGG repeat expansion.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by the University of California, Davis, IACUC.

AUTHOR CONTRIBUTIONS

RW and RB contributed to conception and design of the study. JS, DG-A, AI, and AJ performed behavioral, genetic, and histological assays. JS and RB performed statistical analysis. JS and RB wrote the first draft of the manuscript. JS, DG-A, AJ, AI, RW, and RB contributed sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

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REFERENCES

Allen, N. J., and Lyons, D. A. (2018). Glia as architects of central nervous system formation and function. *Science* 362, 181–185. doi: 10.1126/science.aat0473

Aziz, M., Stathopulu, E., Callias, M., Taylor, C., Turk, J., Oostra, B., et al. (2003). Clinical features of boys with fragile x premutations and intermediate alleles. *Am. J. Med. Genet. B. Neuropsychiatr. Genet.* 121, 119–127. doi: 10.1002/ajmg.b.20030

Baulieu, E. E., Robel, P., and Schumacher, M. (2001). Neurosteroids: Beginning of the story. *Int. Rev. Neurobiol.* 46, 1–32. doi: 10.1016/S0074-7742(01)46057-0

Berman, R. F., Buijsen, R. A., Usdin, K., Pintado, E., Kooy, F., Pretto, D., et al. (2014). Mouse models of the fragile x premutation and fragile x-associated tremor/ataxia syndrome. *J. Neurodev. Disord.* 6:25. doi: 10.1186/1866-1955-6-25

Berman, R. F., and Willemsen, R. (2009). Mouse models of fragile x-associated tremor ataxia. *J. Investig. Med.* 57, 837–841. doi: 10.2310/JIM.0b013e3181af59d6

Berry-Kravis, E., Knox, A., and Hervey, C. (2011). Targeted treatments for fragile x syndrome. *J. Neurodev. Disord* 3, 193–210. doi: 10.1007/s11689-011-9074-7

Boldrini, M., Fulmore, C. A., Tartt, A. N., Simeon, L. R., Pavlova, I., Poposka, V., et al. (2018). Human hippocampal neurogenesis persists throughout aging. *Cell Stem Cell* 22, 589–599e585. doi: 10.1016/j.stem.2018.03.015

Bourgeois, J. A., Seritan, A. L., Casillas, E. M., Hessl, D., Schneider, A., Yang, Y., et al. (2011). Lifetime prevalence of mood and anxiety disorders in fragile x premutation carriers. *J. Clin. Psychiatry* 72, 175–182. doi: 10.4088/JCP.09m05407blu

Brinton, R. D., and Wang, J. M. (2006a). Preclinical analyses of the therapeutic potential of allopregnanolone to promote neurogenesis in vitro and in vivo in transgenic mouse model of alzheimer's disease. *Curr. Alzheimer Res.* 3, 11–17. doi: 10.2174/156720506775697160

Brinton, R. D., and Wang, J. M. (2006b). Therapeutic potential of neurogenesis for prevention and recovery from alzheimer's disease: Allopregnanolone as a proof of concept neurogenic agent. *Curr. Alzheimer Res.* 3, 185–190. doi: 10.2174/156720506777632817

Brouwer, J. R., Huizer, K., Severijnen, L. A., Hukema, R. K., Berman, R. F., Oostra, B. A., et al. (2008). Cgg-repeat length and neuropathological and molecular correlates in a mouse model for fragile x-associated tremor/ataxia syndrome. *J. Neurochem.* 107, 1671–1682. doi: 10.1111/j.1471-4159.2008.05747.x

Cao, Z., Hulsizer, S., Tassone, F., Tang, H. T., Hagerman, R. J., Rogawski, M. A., et al. (2012). Clustered burst firing in fmr1 premutation hippocampal neurons: Amelioration with allopregnanolone. *Hum. Mol. Genet.* 21, 2923–2935. doi: 10.1093/hmg/dds118

Chen, S., Wang, J. M., Irwin, R. W., Yao, J., Liu, L., and Brinton, R. D. (2011). Allopregnanolone promotes regeneration and reduces beta-amyloid burden in a preclinical model of alzheimer's disease. *PLoS One* 6:e24293. doi: 10.1371/journal.pone.0024293

Chen, S., Wang, T., Yao, J., and Brinton, R. D. (2020). Allopregnanolone promotes neuronal and oligodendrocyte differentiation in vitro and in vivo: Therapeutic implication for alzheimer's disease. *Neurotherapeutics* 17, 1813–1824. doi: 10.1007/s13311-020-00874-x

Coffey, S. M., Cook, K., Tartaglia, N., Tassone, F., Nguyen, D. V., Pan, R., et al. (2008). Expanded clinical phenotype of women with the fmr1 premutation. *Am. J. Med. Genet. A* 146A, 1009–1016. doi: 10.1002/ajmg.a.32060

Cornish, K., Kogan, C., Turk, J., Manly, T., James, N., Mills, A., et al. (2005). The emerging fragile x premutation phenotype: Evidence from the domain of social cognition. *Brain Cogn.* 57, 53–60. doi: 10.1016/j.bandc.2004.08.020

D'Hulst, C., Heulens, I., Brouwer, J. R., Willemsen, R., De Geest, N., Reeve, S. P., et al. (2009). Expression of the gabaergic system in animal models for fragile x syndrome and fragile x associated tremor/ataxia syndrome (fxtas). *Brain Res.* 1253, 176–183. doi: 10.1016/j.brainres.2008.11.075

Eriksson, P. S., Perfilieva, E., Bjork-Eriksson, T., Alborn, A. M., Nordborg, C., Peterson, D. A., et al. (1998). Neurogenesis in the adult human hippocampus. *Nat. Med.* 4, 1313–1317. doi: 10.1038/3305

Faroni, A., and Magnaghi, V. (2011). The neurosteroid allopregnanolone modulates specific functions in central and peripheral glial cells. *Front. Endocrinol.* 2:103. doi: 10.3389/fendo.2011.00103

Farzin, F., Perry, H., Hessl, D., Loesch, D., Cohen, J., Bacalman, S., et al. (2006). Autism spectrum disorders and attention-deficit/hyperactivity disorder in boys with the fragile x premutation. *J. Dev. Behav. Pediatr.* 27, S137–S144. doi: 10.1097/00004703-200604002-00012

Garcia-Segura, L. M., Chowen, J. A., and Naftolin, F. (1996). Endocrine glia: Roles of glial cells in the brain actions of steroid and thyroid hormones and in the regulation of hormone secretion. *Front. Neuroendocrinol.* 17, 180–211. doi: 10.1006/frne.1996.0005

Garcia-Segura, L. M., Naftolin, F., Hutchison, J. B., Azcoitia, I., and Chowen, J. A. (1999). Role of astroglia in estrogen regulation of synaptic plasticity and brain repair. *J. Neurobiol.* 40, 574–584. doi: 10.1002/(SICI)1097-4695(19990915)40:4<574::AID-NEU12>3.0.CO;2-8

Genazzani, A. R., Petraglia, F., Bernardi, F., Casarosa, E., Salvestroni, C., Tonetti, A., et al. (1998). Circulating levels of allopregnanolone in humans: Gender, age, and endocrine influences. *J. Clin. Endocrinol. Metab.* 83, 2099–2103. doi: 10.1210/jcem.83.6.4905

Goodrich-Hunsaker, N. J., Wong, L. M., McLennan, Y., Tassone, F., Harvey, D., Rivera, S. M., et al. (2011). Adult female fragile x premutation carriers exhibit age- and cgg repeat length-related impairments on an attentionally based enumeration task. *Front. Hum. Neurosci.* 5:63. doi: 10.3389/fnhum.2011.00063

Gould, E., Reeves, A. J., Fallah, M., Tanapat, P., Gross, C. G., and Fuchs, E. (1999). Hippocampal neurogenesis in adult old world primates. *Proc. Natl. Acad. Sci. U S A* 96, 5263–5267. doi: 10.1073/pnas.96.9.5263

Hagerman, R., Hoem, G., and Hagerman, P. (2010). Fragile x and autism: Intertwined at the molecular level leading to targeted treatments. *Mol. Autism.* 1:12. doi: 10.1186/2040-2392-1-12

Hagerman, R. J., Berry-Kravis, E., Kaufmann, W. E., Ono, M. Y., Tartaglia, N., Lachiewicz, A., et al. (2009). Advances in the treatment of fragile x syndrome. *Pediatrics* 123, 378–390. doi: 10.1542/peds.2008-0317

Hagerman, R. J., Hall, D. A., Coffey, S., Leehey, M., Bourgeois, J., Gould, J., et al. (2008). Treatment of fragile x-associated tremor ataxia syndrome (fxtas) and related neurological problems. *Clin. Interv. Aging* 3, 251–262. doi: 10.2147/CIA.S1794

Hagerman, R. J., Leehey, M., Heinrichs, W., Tassone, F., Wilson, R., Hills, J., et al. (2001). Intention tremor, parkinsonism, and generalized brain atrophy in male carriers of fragile x. *Neurology* 57, 127–130. doi: 10.1212/WNL.57.1.127

Handley, S. L., and Mithani, S. (1984). Effects of alpha-adrenoceptor agonists and antagonists in a maze-exploration model of 'fear'-motivated behaviour. *Naunyn. Schmiedebergs Arch. Pharmacol.* 327, 1–5. doi: 10.1007/BF00504983

Hantash, F. M., Goos, D. M., Crossley, B., Anderson, B., Zhang, K., Sun, W., et al. (2011). Fmr1 premutation carrier frequency in patients undergoing routine population-based carrier screening: Insights into the prevalence of fragile x syndrome, fragile x-associated tremor/ataxia syndrome, and fragile x-associated primary ovarian insufficiency in the united states. *Genet. Med.* 13, 39–45. doi: 10.1097/GIM.0b013e3181fa9fad

Herd, M. B., Belelli, D., and Lambert, J. J. (2007). Neurosteroid modulation of synaptic and extrasynaptic gaba(a) receptors. *Pharmacol. Ther.* 116, 20–34. doi: 10.1016/j.pharmthera.2007.03.007

Holden, H. M., Hoebel, C., Loftis, K., and Gilbert, P. E. (2012). Spatial pattern separation in cognitively normal young and older adults. *Hippocampus* 22, 1826–1832. doi: 10.1002/hipo.22017

Hunsaker, M. R., von Leden, R. E., Ta, B. T., Goodrich-Hunsaker, N. J., Arque, G., Kim, K., et al. (2011). Motor deficits on a ladder rung task in male and female adolescent and adult cgg knock-in mice. *Behav. Brain Res.* 222, 117–121. doi: 10.1016/j.bbr.2011.03.039

Hunsaker, M. R., Wenzel, H. J., Willemsen, R., and Berman, R. F. (2009). Progressive spatial processing deficits in a mouse model of the fragile x premutation. *Behav. Neurosci.* 123, 1315–1324. doi: 10.1037/a0017616

Hunter, J. E., Allen, E. G., Abramowitz, A., Rusin, M., Leslie, M., Novak, G., et al. (2008). Investigation of phenotypes associated with mood and anxiety among male and female fragile x premutation carriers. *Behav. Genet.* 38, 493–502. doi: 10.1007/s10519-008-9214-3

Irwin, R. W., and Brinton, R. D. (2014). Allopregnanolone as regenerative therapeutic for alzheimer's disease: Translational development and clinical promise. *Prog. Neurobiol.* 113, 40–55. doi: 10.1016/j.pneurobio.2013.08.004

Jacquemont, S., Farzin, F., Hall, D., Leehey, M., Tassone, F., Gane, L., et al. (2004). Aging in individuals with the fmr1 mutation. *Am. J. Ment. Retard* 109, 154–164. doi: 10.1352/0895-8017(2004)109<154:AIIWTF>2.0.CO;2

Johansson, I. M., Birzniece, V., Lindblad, C., Olsson, T., and Backstrom, T. (2002). Allopregnanolone inhibits learning in the morris water maze. *Brain Res.* 934, 125–131. doi: 10.1016/S0006-8993(02)02414-9

Johansson, M., Stromberg, J., Ragagnin, G., Doverskog, M., and Backstrom, T. (2016). Gabaa receptor modulating steroid antagonists (gamsa) are functional in vivo. *J. Steroid Biochem. Mol. Biol.* 160, 98–105. doi: 10.1016/j.jsbmb.2015.10.019

Jordan, C. L. (1999). Glia as mediators of steroid hormone action on the nervous system: An overview. *J. Neurobiol.* 40, 434–445. doi: 10.1002/(SICI)1097-4695(19990915)40:4<434::AID-NEU2>3.0.CO;2-T

Kempermann, G., Gast, D., and Gage, F. H. (2002). Neuroplasticity in old age: Sustained fivefold induction of hippocampal neurogenesis by long-term environmental enrichment. *Ann. Neurol.* 52, 135–143. doi: 10.1002/ana.10262

Kempermann, G., Kuhn, H. G., and Gage, F. H. (1997). Genetic influence on neurogenesis in the dentate gyrus of adult mice. *Proc. Natl. Acad. Sci. U S A* 94, 10409–10414. doi: 10.1073/pnas.94.19.10409

Kim, M. T., Soussou, W., Gholmieh, G., Ahuja, A., Tanguay, A., Berger, T. W., et al. (2006). 17beta-estradiol potentiates field excitatory postsynaptic potentials within each subfield of the hippocampus with greatest potentiation of the associational/commissural afferents of ca3. *Neuroscience* 141, 391–406. doi: 10.1016/j.neuroscience.2006.03.075

Koldewyn, K., Hessl, D., Adams, J., Tassone, F., Hagerman, P. J., Hagerman, R. J., et al. (2008). Reduced hippocampal activation during recall is associated with elevated fmr1 mrna and psychiatric symptoms in men with the fragile x premutation. *Brain Imaging Behav.* 2, 105–116. doi: 10.1007/s11682-008-9020-9

Kuhn, H. G., Dickinson-Anson, H., and Gage, F. H. (1996). Neurogenesis in the dentate gyrus of the adult rat: Age-related decrease of neuronal progenitor proliferation. *J. Neurosci.* 16, 2027–2033. doi: 10.1523/JNEUROSCI.16-06-02027.1996

Moore, C. J., Daly, E. M., Schmitz, N., Tassone, F., Tysoe, C., Hagerman, R. J., et al. (2004). A neuropsychological investigation of male premutation carriers of fragile x syndrome. *Neuropsychologia* 42, 1934–1947. doi: 10.1016/j.neuropsychologia.2004.05.002

Napoli, E., Schneider, A., Wang, J. Y., Trivedi, A., Carrillo, N. R., Tassone, F., et al. (2019). Allopregnanolone treatment improves plasma metabolomic profile associated with gaba metabolism in fragile x-associated tremor/ataxia syndrome: A pilot study. *Mol. Neurobiol.* 56, 3702–3713. doi: 10.1007/s12035-018-1330-3

Nichols, N. R. (1999). Glial responses to steroids as markers of brain aging. *J. Neurobiol.* 40, 585–601. doi: 10.1002/(SICI)1097-4695(19990915)40:4<585::AID-NEU13>3.0.CO;2-1

Nilsen, J., Irwin, R. W., Gallaher, T. K., and Brinton, R. D. (2007). Estradiol in vivo regulation of brain mitochondrial proteome. *J. Neurosci.* 27, 14069–14077. doi: 10.1523/JNEUROSCI.4391-07.2007

Nixon, K., Kim, D. H., Potts, E. N., He, J., and Crews, F. T. (2008). Distinct cell proliferation events during abstinence after alcohol dependence: Microglia proliferation precedes neurogenesis. *Neurobiol. Dis.* 31, 218–229. doi: 10.1016/j.nbd.2008.04.009

Noorbakhsh, F., Ellestad, K. K., Maingat, F., Warren, K. G., Han, M. H., Steinman, L., et al. (2011). Impaired neurosteroid synthesis in multiple sclerosis. *Brain* 134, 2703–2721. doi: 10.1093/brain/awr200

Rietze, R., Poulin, P., and Weiss, S. (2000). Mitotically active cells that generate neurons and astrocytes are present in multiple regions of the adult mouse hippocampus. *J. Comp. Neurol.* 424, 397–408. doi: 10.1002/1096-9861(20000828)424:3<397::AID-CNE2>3.0.CO;2-A

Rodriguez-Revenga, L., Madrigal, I., Pagonabarraga, J., Xuncla, M., Badenas, C., Kulisevsky, J., et al. (2009). Penetrance of fmr1 premutation associated pathologies in fragile x syndrome families. *Eur. J. Hum. Genet.* 17, 1359–1362. doi: 10.1038/ejhg.2009.51

Shickman, R., Famula, J., Tassone, F., Leehey, M., Ferrer, E., Rivera, S. M., et al. (2018). Age- and cgg repeat-related slowing of manual movement in fragile x carriers: A prodrome of fragile x-associated tremor ataxia syndrome? *Mov. Disord* 33, 628–636. doi: 10.1002/mds.27314

Shoji, H., and Miyakawa, T. (2019). Age-related behavioral changes from young to old age in male mice of a c57bl/6j strain maintained under a genetic stability program. *Neuropsychopharmacol. Rep.* 39, 100–118. doi: 10.1002/npr2.12052

Singh, C., Liu, L., Wang, J. M., Irwin, R. W., Yao, J., Chen, S., et al. (2012). Allopregnanolone restores hippocampal-dependent learning and memory and neural progenitor survival in aging 3xtgad and nontg mice. *Neurobiol Aging* 33, 1493–1506. doi: 10.1016/j.neurobiolaging.2011.06.008

Snyder, J. S., Choe, J. S., Clifford, M. A., Jeurling, S. I., Hurley, P., Brown, A., et al. (2009). Adult-born hippocampal neurons are more numerous, faster maturing, and more involved in behavior in rats than in mice. *J. Neurosci.* 29, 14484–14495. doi: 10.1523/JNEUROSCI.1768-09.2009

Sukoff Rizzo, S. J., Anderson, L. C., Green, T. L., McGarr, T., Wells, G., and Winter, S. S. (2018). Assessing healthspan and lifespan measures in aging mice: Optimization of testing protocols, replicability, and rater reliability. *Curr. Protoc. Mouse Biol.* 8:e45. doi: 10.1002/cpmo.45

Sun, C., Ou, X., Farley, J. M., Stockmeier, C., Bigler, S., Brinton, R. D., et al. (2012). Allopregnanolone increases the number of dopaminergic neurons in substantia nigra of a triple transgenic mouse model of alzheimer's disease. *Curr. Alzheimer Res.* 9, 473–480. doi: 10.2174/156720512800492567

Tassone, F., Iong, K. P., Tong, T. H., Lo, J., Gane, L. W., Berry-Kravis, E., et al. (2012). Fmr1 cgg allele size and prevalence ascertained through newborn screening in the united states. *Genome Med.* 4:100. doi: 10.1186/gm401

Thomas, A., Burant, A., Bui, N., Graham, D., Yuva-Paylor, L. A., and Paylor, R. (2009). Marble burying reflects a repetitive and perseverative behavior more than novelty-induced anxiety. *Psychopharmacology* 204, 361–373. doi: 10.1007/s00213-009-1466-y

Tuncdemir, S. N., Lacefield, C. O., and Hen, R. (2019). Contributions of adult neurogenesis to dentate gyrus network activity and computations. *Behav. Brain Res.* 374:112112. doi: 10.1016/j.bbr.2019.112112

Turkmen, S., Backstrom, T., Wahlstrom, G., Andreen, L., and Johansson, I. M. (2011). Tolerance to allopregnanolone with focus on the gaba-a receptor. *Br. J. Pharmacol.* 162, 311–327. doi: 10.1111/j.1476-5381.2010.01059.x

Van Dam, D., Errijgers, V., Kooy, R. F., Willemsen, R., Mientjes, E., Oostra, B. A., et al. (2005). Cognitive decline, neuromotor and behavioural disturbances in a mouse model for fragile-x-associated tremor/ataxia syndrome (fxtas). *Behav. Brain Res.* 162, 233–239. doi: 10.1016/j.bbr.2005.03.007

Walf, A. A., and Frye, C. A. (2007). The use of the elevated plus maze as an assay of anxiety-related behavior in rodents. *Nat. Protoc.* 2, 322–328. doi: 10.1038/nprot.2007.44

Wang, J. M., Johnston, P. B., Ball, B. G., and Brinton, R. D. (2005). The neurosteroid allopregnanolone promotes proliferation of rodent and human neural progenitor cells and regulates cell-cycle gene and protein expression. *J. Neurosci.* 25, 4706–4718. doi: 10.1523/JNEUROSCI.4520-04.2005

Wang, J. M., Liu, L., Irwin, R. W., Chen, S., and Brinton, R. D. (2008). Regenerative potential of allopregnanolone. *Brain Res. Rev.* 57, 398–409. doi: 10.1016/j.brainresrev.2007.08.010

Wang, J. M., Singh, C., Liu, L., Irwin, R. W., Chen, S., Chung, E. J., et al. (2010). Allopregnanolone reverses neurogenic and cognitive deficits in mouse model of alzheimer's disease. *Proc. Natl. Acad. Sci. U S A* 107, 6498–6503. doi: 10.1073/pnas.1001422107

Wang, J. Y., Trivedi, A. M., Carrillo, N. R., Yang, J., Schneider, A., Giulivi, C., et al. (2017). Open-label allopregnanolone treatment of men with fragile x-associated tremor/ataxia syndrome. *Neurotherapeutics* 14, 1073–1083. doi: 10.1007/s13311-017-0555-6

Wenzel, H. J., Murray, K. D., Haify, S. N., Hunsaker, M. R., Schwartzer, J. J., Kim, K., et al. (2019). Astroglial-targeted expression of the fragile x cgg repeat premutation in mice yields ran translation, motor deficits and possible evidence for cell-to-cell propagation of fxtas pathology. *Acta Neuropathol. Commun.* 7:27. doi: 10.1186/s40478-019-0677-7

Willemsen, R., Hoogeveen-Westerveld, M., Reis, S., Holstege, J., Severijnen, L. A., Nieuwenhuizen, I. M., et al. (2003). The fmr1 cgg repeat mouse displays ubiquitin-positive intranuclear neuronal inclusions; implications for the cerebellar tremor/ataxia syndrome. *Hum. Mol. Genet.* 12, 949–959. doi: 10.1093/hmg/ddg114

Wojtowicz, J. M., and Kee, N. (2006). Brdu assay for neurogenesis in rodents. *Nat. Protoc.* 1, 1399–1405. doi: 10.1038/nprot.2006.224

Yang, T. T., Lo, C. P., Tsai, P. S., Wu, S. Y., Wang, T. F., Chen, Y. W., et al. (2015). Aging and exercise affect hippocampal neurogenesis via different mechanisms. *PLoS One* 10:e0132152. doi: 10.1371/journal.pone.0132152

Zhao, C., Teng, E. M., Summers, R. G. Jr., Ming, G. L., and Gage, F. H. (2006). Distinct morphological stages of dentate granule neuron maturation in the adult mouse hippocampus. *J. Neurosci.* 26, 3–11. doi: 10.1523/JNEUROSCI.3648-05.2006

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Experiences of the Molecular Diagnosis of Fragile X Syndrome in Ecuador

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Fragile X syndrome (FXS) is the most common cause of hereditary intellectual disability and the second most common cause of intellectual disability of genetic etiology. This complex neurodevelopmental disorder is caused by an alteration in the CGG trinucleotide expansion in fragile X mental retardation gene 1 (*FMR1*) leading to gene silencing and the subsequent loss of its product: fragile X mental retardation protein 1 (FMRP). Molecular diagnosis is based on polymerase chain reaction (PCR) screening followed by Southern blotting (SB) or Triplet primer-PCR (TP-PCR) to determine the number of CGG repeats in the *FMR1* gene. We performed, for the first time, screening in 247 Ecuadorian male individuals with clinical criteria to discard FXS. Analysis was carried out by the Genetics Service of the Hospital de Especialidades No. 1 de las Fuerzas Armadas (HE-1), Ecuador. The analysis was performed using endpoint PCR for CGG fragment expansion analysis of the *FMR1* gene. Twenty-two affected males were identified as potentially carrying the full mutation in FMR1 and thus diagnosed with FXS that is 8.1% of the sample studied. The average age at diagnosis of the positive cases was 13 years of age, with most cases from the geographical area of Pichincha (63.63%). We confirmed the familial nature of the disease in four cases. The range of CGG variation in the population was 12–43 and followed a modal distribution of 27 repeats. Our results were similar to those reported in the literature; however, since it was not possible to differentiate between premutation and mutation cases, we can only establish a molecular screening approach to identify an expanded CGG repeat, which makes it necessary to generate national strategies to optimize molecular tests and establish proper protocols for the diagnosis, management, and follow-up of patients, families, and communities at risk of presenting FXS.

Keywords: dynamic mutation diseases, fragile X syndrome, FMR1, repetitions, intellectual disabilities

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INTRODUCTION

Fragile X syndrome (FXS) is the most common cause of hereditary intellectual disability (1). The syndrome has a complex clinical course, and patients show a unique phenotype that includes an elongated face, pointed ears, high palate, hyperlaxity, etc. Children with FXS also exhibit behavioral characteristics including anxiety, irritability, aggressiveness, and compulsivity (2). In approximately

60% of cases, patients are also diagnosed with autism spectrum disorder (ASD) (3), representing the most common monogenic cause of ASD. Autism, intellectual disability, and behavioral disorders develop at a lower rate and to a much lesser degree in females than in males (4).

FXS is caused by an abnormal expansion of a CGG triplet, greater than 200 repeats in the promoter region of the *FMR1* gene located at Xq27.3 (5, 6). This expansion causes a loss of function that prevents the encoding of FMRP, a protein involved in several brain development and function processes (7). The *FMR1* gene contains 17 exons (38kb) and the mutation normally occurs in intron 10, which contains the gene's unstable region (8). According to the reported expansion number, the allele can be classified into normal alleles, with 6–44 CGG repeats, premutation (PM) alleles, with 55–200 CGG repeats, and full mutation (FM) alleles with more than 200 CGG repeats. Intermediate or gray zone alleles are those harboring between 45 and 54 CGG repeats and are considered precursors of the PM allele (9).

Methods to diagnostic FXS include molecular tests such as PCR that allows determining if there is a CGG expansion, which, in combination with SB, allows determining the exact number of CGG repeats and the degree of methylation of the *FMR1* gene (10), and triplet-primed PCR assay that detects *FMR1* alleles throughout the expanded range (11). On the other hand, immunohistochemical tests can be performed to determine the presence or absence of the FMRP protein (12).

The frequency for the FM has been estimated to be higher in males than in females; internationally, the prevalence is 1/4,000 in males and 1/8,000 in females, and the PM frequency for this gene is 1/259 in females and 1/379 in male carriers (13). There is some variation depending on the population studied, e.g., in the United States, the prevalence of the FM in European descendants is 1/3,717, and in African descendants, the prevalence is 1/2,545 males (14, 15). The prevalence of carriers of the PM in females is 1/151, and for males, it is 1/468, while for carriers of intermediate or "gray zone" alleles, it is 1/35 in females and 1/42 in males (16).

The prevalence of FXS in Ecuador remains uncertain. Most diagnoses of FXS have been made in isolation through screening projects. Of Ecuador's 17 million inhabitants, 2.72% (472,213 inhabitants) are known to have some type of disability, of which 23% (108,588 inhabitants) are cognitive or intellectual (17). The elevated number of undiagnosed patients with intellectual disabilities in our country highlights the need to implement molecular study tools to characterize the population in order to improve clinical assessment and genetic counseling for patients and their families. This work describes the experience in the molecular diagnostic approach of fragile X in the HE-1 in Ecuador.

MATERIALS AND METHODS

This is a genetic, descriptive, cross-sectional, retrospective study of 247 male patients to rule out FXS, conducted at the Genetic Service of HE-1 in Quito, Ecuador. These patients were referred for genetic evaluation between 2011 and 2021, from different institutions belonging to the Comprehensive Public Health Network.

Clinical and genetic data were extracted from the patients' physical medical records, the clinical notes reported in the HE-1 hospital management system, the referral sheets, and the record books of the molecular biology laboratory. Excel spreadsheets were used for data processing, followed by a descriptive analysis using SPSS to obtain measures of central tendency for the quantitative variables and the absolute frequency, relative frequency, and relative percentage frequency for qualitative variables.

Participant Inclusion Criteria

Men with a diagnosis of intellectual disability, ASD, language delay, or a positive family history of FXS.

DNA Extraction

DNA used for PCR amplification was extracted from peripheral blood leukocytes by High Pure PCR Template Preparation Kit (Roche GmbH, Mannheim, Germany) according to the manufacturer's protocol. DNA quantification was performed to ensure the quality of the DNA extraction and to determine if the nucleic acid concentration was optimal for the study (∼50 ng).

PCR-Based Method

Amplification of normal and expanded alleles was performed by endpoint PCR using the Veriti Thermal Cycler (Applied Biosystems Inc., California, USA).

Fast Start High Fidelity PCR System Kit (Roche Gmb., Mannheim, Germany) was used to assemble the PCR and two sets of oligonucleotide primers were used to amplify the repetitive CGG region of the *FMR1* gene: Fx-C: GCTCAGCTCCGTTTCGGTTTCACTTCCGGT and Fx-F: AGCCCCGCACTTCCACCACCAGCTCCTCCA, owned by Gene Link Inc. (New York, USA).

The PCR assay was performed in a 48-µl reaction volume containing about 200 ng of genomic DNA, 18 mmol/L of $MgCl_2$, 0.625 U/ml of DMSO, 0.36 mmol/L of dNTPs, and 0.83 µmol/L of the pair of primers for PCR amplification of the CGG repetitive region of the *FMR1* gene (Gene Link, INC, USA) and 5 units/reaction of Taq polymerase (ROCHE; Hamburg, Germany). The reactions were denaturated for 10 min at 98°C, after which the Taq Polymerase was added; followed by 10 cycles consisting of 97°C for 30 s, 65°C for 45 s, and 68°C for 4 min; and 20 cycles consisting of 97°C for 30 s, 65°C for 45 s, 68°C for 4 min 20 s, and finally a 10-min extension at 68°C (**Table 1**). The PCR-amplified products were separated by 3% agarose gel electrophoresis using a 50-bp DNA Step Ladder as a molecular marker, Blue/Orange Loading Dye [6X] in the loading mix, and fluorescent Diamond Nucleic Acid Dye (Promega, Wisconsin, USA).

RESULTS

Resource Identification Initiative

Two hundred and forty-seven men referred for genetic evaluation were screened for FXS. Of these, 22 patients were

identified to have an abnormal CGG expansion of the FMR1 gene (8.1%); 46% came from the Pichincha Province (**Table 1**). Family history of the condition was detected in 10 patients (four families). The average age of diagnosis of the positive cases was 13 years, with age range between 2 and 31 years old. Cases were found in 6 of Ecuador's 24 provinces, with the Pichincha Province, capital of Ecuador, presenting the highest number of positive results: 14 cases, 63.63% (**Figure 1**). The average expansion and the number of CGG repeats for male Ecuadorian patients, with some clinical criteria studied to discard FXS, were 12–43 (**Figure 3**).

TABLE 1 | Endpoint PCR program for fragile X syndrome.

Step	Time and temperature	Cycles
Denaturation	10 min at 98°C	1
Hold	5 min (pause) at 98°C	1
Add Taq enzyme mix while on hold		
Denaturation	30 s at 97°C	10
Annealing	45 s at 65°C	
Extension	4 min at 68°C	
Denaturation	30 s at 97°C	20
Annealing	45 s at 65°C	
Extension	4 min 20 s at 68°C	
Fill up	10 min at 68°C	1
Hold	Hold for infinity at 4°C	1

The absence of DNA fragments in the agarose gel indicates that the allele was so expanded that it cannot be observed given the limited sensitivity of the technique (100–120 CGG repeats). This corresponds to a positive result, which is confirmed considering the positive clinical elements (family history, intellectual disability, attention deficit and hyperactivity, autism spectrum, long narrow face, prominent jaw and ears, and joint hypermobility). In addition, this result is validated by performing real-time PCR to ensure adequate amplification of the genetic material (**Figure 2**).

Translation Results

The average range of CGG repeats in individuals with a normal expansion in FMR1 was 27 repeats (**Figure 3**). PM is not included since the technique does not allow establishing the exact number of repeats in overexpanded affected alleles. The protocol only allows a normal repetition range to be established. If the value exceeds that range, the allele is not observed in the electrophoretic run, ensuring its overexpansion but without exact determination of the size.

DISCUSSION

FXS is the most common monogenic condition underlying ASD and intellectual disability (18). For this reason, the American College of Medical Genetics and Genomics recommends FXS testing for all individuals with developmental delay, intellectual disability, and/or behavioral problems (19).

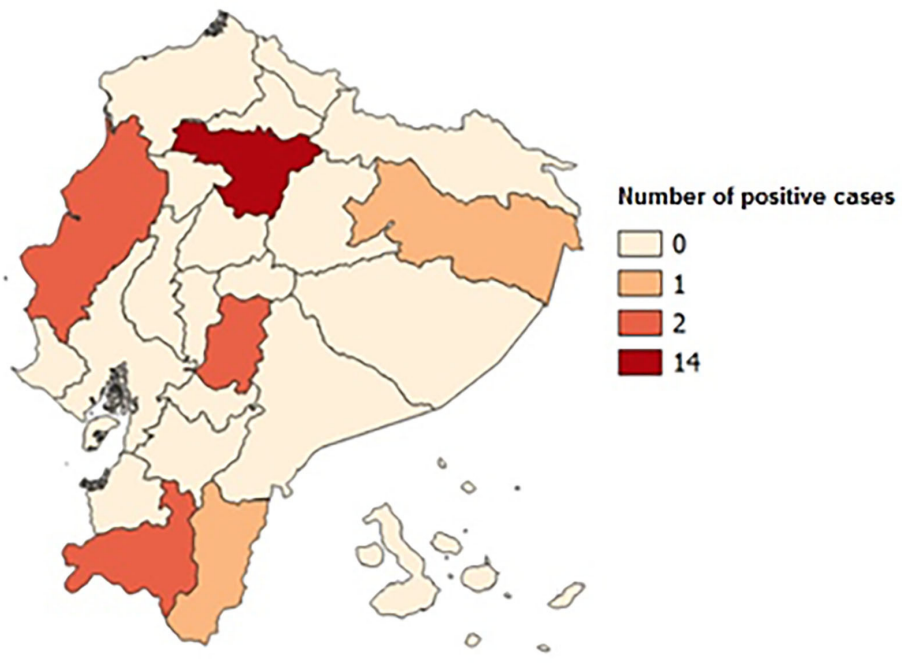

FIGURE 1 | Map of Ecuador with positive case distribution by provinces. The Pichincha Province has 14 positive cases; the Guayas, Loja, and Chimborazo Provinces have 2; and the Orellana and Zamora Chinchipe Provinces have 1 positive case.

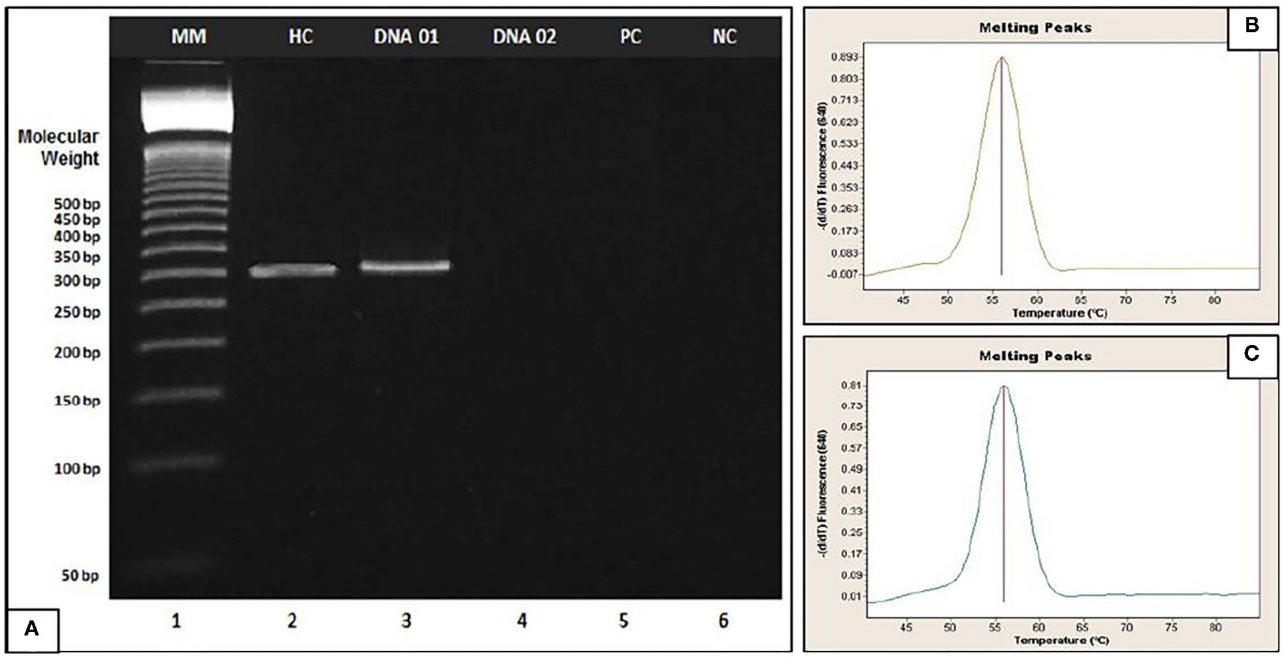

FIGURE 2 | (A) Agarose gel electrophoresis of fragile X. Example applied to patients (DNA 01 and DNA 02). Lane 1 (MM): 50-bp DNA Step Ladder, Lane 2: Healthy Control (HC), Lane 3 (DNA 01): Healthy patient, Lane 4 (DNA 02): Affected patient, Lane 5: Positive Control (PC) and Lane 6: Negative Control (CN). **(B,C)** Amplification of genetic material by Real-Time PCR of DNA 02 and Positive Control respectively; DNA 02 and Positive control were quantified in Quantus Fluorometer (Promega, Wisconsin, USA) and concentration values between 65 and 75 ng/μl were obtained.

Estimation of prevalence varies depending on the population studied and the diagnostic test used. Of the 247 Ecuadorian patients with intellectual disabilities and/or behavior problems who participated in this study, 8.1% were found to be positive through the application of molecular screening of PCR, establishing a reference point in our country, compared to previous studies that describe the presence of this condition in 7% to 8% of children with ASD (20).

Family segregation was identified in 4 families, which is justified by the inheritance mechanism, especially if a recurrent family history, an X-linked pattern, or a phenotype compatible with this disorder is considered (21). The average age of diagnosis in the patients seen in the laboratory was 13 years, much older than the age of diagnosis described in other studies, such as that described by Bailey et al., where the average age of diagnosis of FXS in children remained relatively stable over the 7-year period, at ∼35 to 37 months (22). Among the studies carried out in the region, Colombia and Chile also show a higher age of diagnosis than expected (23, 24). The difference found in our data and in neighboring countries may be explained by the limited access to the health system and the lack of national protocols for molecular study of this condition in people with intellectual disabilities.

Most of the positive results were found in the Pichincha province in the Andes, possibly because it is the region of the country with the largest number of tertiary hospitals with access to molecular testing. In an ancestry analysis study in the Ecuadorian population carried out by Zambrano and collaborators, Ecuador's genetic composition is described as a mixture of three groups of ancestors: Native Americans—the largest proportion in all regions (over 50%)—and a lower proportion of people of European and African descent. The proportion of Native American ancestry in the Andes region is 64.7%, European ancestry in the same region reaches 26.8%, while African ancestry reaches 0.85% (25). Peprah describes the lack of information on *FMR1* gene mutations in most countries with a non-European population (26). Further studies are needed to find out the extent of the mutation of the gene in the Pichincha Province.

The proportion of patients with FXS who came for genetic evaluation is low (2.7% of the total cases studied for rare diseases in HE1 since 2010) in relation to other diseases also diagnosed in the service such as Steinert myotonic dystrophy (12.2% of the cases studied) or Huntington's chorea (8.2% of the cases studied). This may be due to the lack of referral of patients with FXS and indicates a need for education of health professionals from different disciplines regarding the importance of early detection, which could lead to minimizing or preventing the risk of transmission.

In negative cases, there was a multimodal distribution of 27 (8.90%), 26 (7.28%), 29 (7.04%), and 35 (7.04%) repeats, similar to that reported in different populations worldwide where the allelic distribution of the expansion and the number of CGG repeats of the gene is between 29 and 30 (26) (**Figure 3**). The data are similar in studies conducted in Brazil (27), Cameroon (28), Chile (29), China and India (30), United States (31), and Mexico (32). The small differences found (one or two repeats of

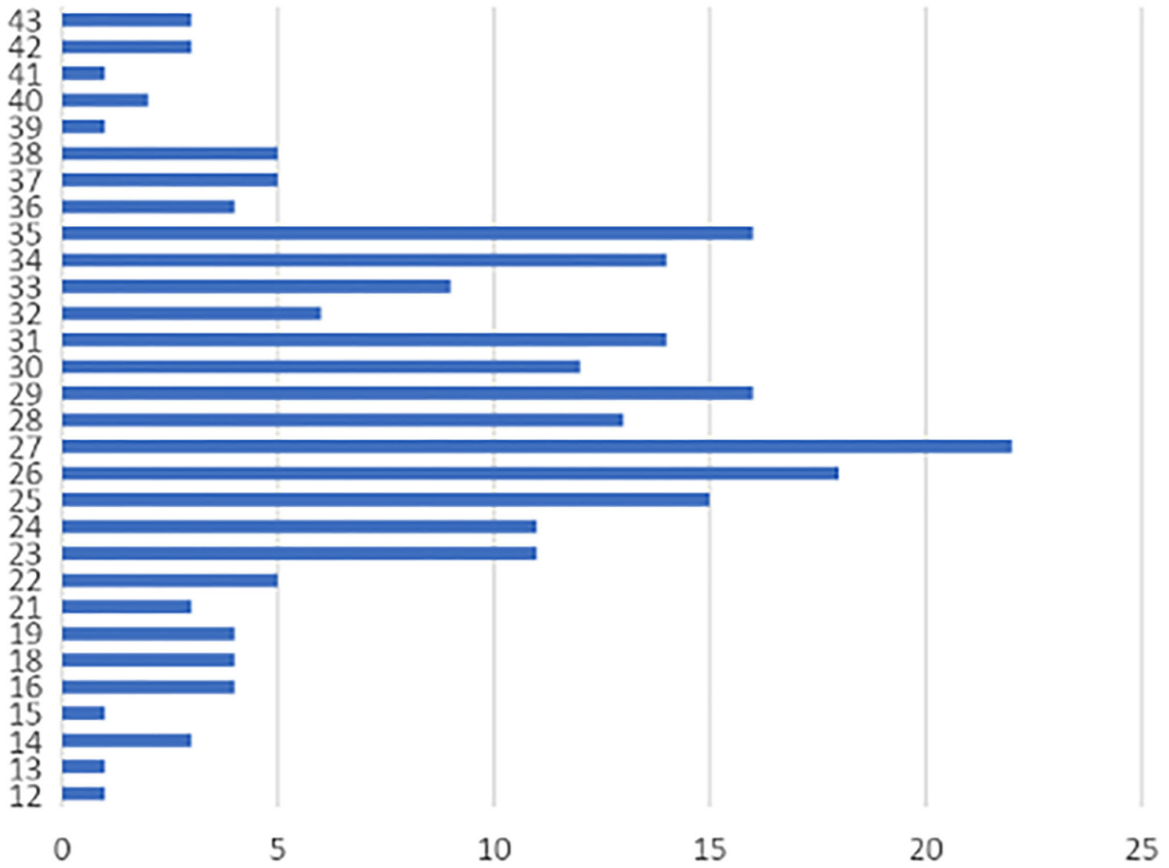

FIGURE 3 | Analysis of CGG repeats in the normal unaffected population of Ecuador. Horizontal axis (X axis): CGG repeats, Vertical axis (Y axis): Frequency in the number of CGG repetitions. The range of variation of CGG in the population is 12–43 and shows a modal distribution of 27 repeats (8.90% of case). Other important percentages of frequency were of 26 repeats (7.28%), and 29 and 35 repeats (7.04%).

CGG) are related to experimental errors given by differences in the precision of the techniques used and the lack of their own population patterns (33), in addition to population and ethnic characteristics (34).

Limitations

The standard procedure for the diagnosis of FXS is PCR and Southern blot, and results can only be analyzed with a gene analyzer or capillary electrophoresis equipment. Unfortunately, these tools were not available in the Medical Genetics service; therefore, only endpoint PCR and agarose gel electrophoresis were used. The absence of DNA fragments in the agarose gel indicated that the allele was expanded and was not observable due to the limited sensitivity of this technique (100–120 CGG repeats). In this case, the result was validated by real-time PCR to ensure adequate amplification of genetic material. In addition, the technique used cannot detect gene PM.

In addition, the sensitivity of the technique does not allow to visualize the second allele, corresponding to the homologous X chromosome in a woman; thus, there are two possibilities: two superimposed alleles with similar molecular weights. In this case, the patient would present a normal phenotype, without risk of transmission of FXS, or that the second allele had a large number of CGG repeats that cannot be amplified by the PCR. In this case, the patient would be a carrier of a PM or a complete mutation of the *FMR1* gene, with the risk of transmitting FXS to her descendants. To avoid confusion, this screening was not performed in women.

CONCLUSION

Using the endpoint PCR technique, it is not possible to differentiate the cases of mutation carriers from those of PM, so it does not allow us to obtain a real rate of patients with the condition. It only allows us to make an approximation of the possible cases, functioning as an initial screening technique. Although our values were similar to those reported in the literature, as we were unable to differentiate the number of expansions, we could be overestimating the number of cases with a diagnosis of FXS. National strategies should be implemented to optimize molecular tests and establish proper protocols for the diagnosis to implement the use of diagnostic techniques such

as SB and triplet-primed PCR assay, guides for management, and follow-up of patients, families, and communities at risk of presenting FXS.

The limitation of the study is the laboratory technique. The endpoint PCR and agarose gel electrophoresis used in this study do not allow to differentiate the premutated alleles (55 to 200 CGG repeats) and complete mutation alleles (>200 CGG repeats). These are important characteristics to understand the inheritance mechanism at the family (35). In addition to PCR, Southern blot and capillary electrophoresis are standard techniques for the diagnosis of FXS that allow confirmation of premutated allele carriers and full mutation. The laboratory does not have a capillary electrophoresis equipment because it is a complex and expensive technique. Instead, the Fast Start High Fidelity PCR System kit (Roche GmbH, Mannheim, Germany) was used to assemble the PCR and agarose gel electrophoresis. This study helps in the screening of the frequency of patients with FXS in Ecuador and makes visible the need to implement molecular diagnosis for people with intellectual disabilities and learning disorders.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

Ethical review and approval was not required for the study on human participants in accordance with the Local Legislation and Institutional Requirements. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JP-P, EO, AL-C, and JV drafted the manuscript. AL-P, CR, and EO permormed testing and data collection.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.716311/full#supplementary-material

REFERENCES

1. Brun GC, Artigas PJ. Aspectos psicolingüísticos en el síndrome del cromosoma X frágil. *Revista de Neurología.* (2001) 33:29–32. doi: 10.33588/rn.33S1.2001347
2. Hersh JH, Saul RA. Health supervision for children with fragile X syndrome. *Pediatrics.* (2011) 127:994–1006. doi: 10.1542/peds.2010-3500
3. Reddy KS. Cytogenetic abnormalities and fragile-X syndrome in Autism Spectrum Disorder. *BMC Med Genet.* (2005) 6:1–16. doi: 10.1186/1471-2350-6-3
4. Harris SW, Hessl D, Goodlin-Jones B, Ferranti J, Bacalman S, Barbato I, et al. Autism profiles of males with fragile X syndrome. *Am J Mental Retard.* (2008) 113:427–38. doi: 10.1352/2008.113:427-438
5. Devys D, Biancalana V, Rousseau F, Boué J, Mandel JL, Oberlé I. Analysis of full fragile X mutations in fetal tissues and monozygotic twins indicate that abnormal methylation and somatic heterogeneity are established early in development. *Am J Med Genet.* (1992) 43:208–16. doi: 10.1002/ajmg.1320430134
6. van Eyk C L, Richards RI. *Dynamic Mutations. Tandem Repeat Polymorphisms.* New York: Springer (2012). p. 55–77. doi: 10.1007/978-1-4614-5434-2_5
7. Richter JD, Zhao X. The molecular biology of FMRP: new insights into fragile X syndrome. *Nat Rev Neurosci.* (2021) 22:209–22. doi: 10.1038/s41583-021-00432-0
8. Eichler EE, Richards CS, Gibbs RA, Nelson DL. Fine structure of the human FMR1 gene. *Hum Mol Genet.* (1993) 2:1147–53. doi: 10.1093/hmg/2.8.1147
9. Tzeng CC, Tsai LP, Hwu WL, Lin SJ, Chao MC, Jong YJ, et al. Prevalence of the FMR1 mutation in Taiwan assessed by large-scale screening of newborn boys and analysis of DXS548-FRAXAC1 haplotype. *Am J Med Genet Part A.* (2005) 133:37–43. doi: 10.1002/ajmg.a.30528
10. Lyon E, Laver T, Yu P, Jama M, Young K, Zoccoli M, et al. simple, high-throughput assay for Fragile X expanded alleles using triple repeat primed PCR and capillary electrophoresis. *J Molec Diagnost.* (2010) 12:505–11. doi: 10.2353/jmoldx.2010.090229
11. Tassone F. Advanced technologies for the molecular diagnosis of fragile X syndrome. *Expert Rev Mol Diagn.* (2015) 15:1465–73. doi: 10.1586/14737159.2015.1101348
12. LaFauci G, Adayev T, Kascsak R, Brown WT. Detection and quantification of the fragile X mental retardation protein 1 (FMRP). *Genes.* (2016) 7:121. doi: 10.3390/genes7120121
13. Mazzocco MM. Advances in research on the fragile X syndrome. *Ment Retard Dev Disabil Res Rev.* (2000) 6:96–106. doi: 10.1002/1098-2779(2000)6:2<96::AID-MRDD3>3.0.CO;2-H
14. Youings S, Murray A, Dennis N, Ennis S, Lewis C, McKechnie N, et al. FRAXA and FRAXE: the results of a five year survey. *J Med Genet.* (2000) 37:415–21. doi: 10.1136/jmg.37.6.415
15. Crawford DC, Meadows KL, Newman JL, Taft LF, Scott E, Leslie M, et al. Prevalence of the fragile X syndrome in African-Americans. *Am J Med Genet.* (2002) 110:226–33. doi: 10.1002/ajmg.10427
16. Seltzer MM, Baker MW, Hong J, Maenner M, Greenberg J, Mandel D. Prevalence of CGG Expansions of the FMR1 Gene in a US Population-Based Sample. *Am J Med Genet.* (2012) 159:589–97. doi: 10.1002/ajmg.b.32065
17. Consejo Nacional para la Igualdad de Discapacidades. Estadísticas de Discapacidad [Internet]. (2021) Available online at: https://www.consejodiscapacidades.gob.ec/estadisticas-de-discapacidad/. (accessed May 18, 2021)
18. Berry-Kravis EM, Lindemann L, Jønch AE, Apostol G, Bear MF, Carpenter RL, et al. Drug development for neurodevelopmental disorders: lessons learned from fragile X syndrome. *Nat Rev Drug Discovery.* (2018) 17:280–99. doi: 10.1038/nrd.2017.221
19. Monaghan KG, Lyon E, Spector EB, ACMG. Standards and Guidelines for fragile X testing: a revision to the disease-specific supplements to the Standards and Guidelines for Clinical Genetics Laboratories of the American

College of Medical Genetics and Genomics. *Genet Med.* (2013) 15:575–86. doi: 10.1038/gim.2013.61
20. Muhle R, Trentacoste SV, Rapin I. The genetics of autism. *Pediatrics.* (2004) 113:e472–86. doi: 10.1542/peds.113.5.e472
21. Spector EB. *Fragile X—A Family of Disorders: Changing Phenotype and Molecular Genetics*. In The Principles of Clinical Cytogenetics. New York: Springer (2013). p. 453–71. doi: 10.1007/978-1-4419-1688-4_19
22. Bailey DB, Raspa M, Bishop E, Holiday D. No change in the age of diagnosis for fragile x syndrome: findings from a national parent survey. *Pediatrics.* (2009) 124:527–33. doi: 10.1542/peds.2008-2992
23. Saldarriaga-Gil W, Cabal-Herrera AM, Fandiño-Losada A, Vásquez A, Hagerman R, Tassone F. Inequities in diagnosis of Fragile X syndrome in Colombia. *J Appl Res Intellect Disabil.* (2021) 34:830–9. doi: 10.1111/jar.12863
24. Santa María L, Aliaga S, Faundes V, Morales P, Pugin Á, Curotto B, et al. FMR1 gene mutations in patients with fragile X syndrome and obligate carriers: 30 years of experience in Chile. *Genet Res (Camb).* (2016) 98:e11. doi: 10.1017/S0016672316000082
25. Zambrano AK, Gaviria A, Cobos-Navarrete S, Gruezo C, Rodríguez-Pollit C, Armendáriz-Castillo I, et al. The three-hybrid genetic composition of an Ecuadorian population using AIMs-InDels compared with autosomes, mitochondrial DNA and Y chromosome data. *Sci Rep.* (2019) 9:1–8. doi: 10.1038/s41598-019-45723-w
26. Peprah E. Fragile X syndrome: the FMR1 CGG repeat distribution among world populations. *Ann Hum Genet.* (2012) 76:178–91. doi: 10.1111/j.1469-1809.2011.00694.x
27. Mingroni-Netto RC, Angeli CB, Auricchio MTBM, Leal-Mesquita ER, Ribeiro-dos-Santos AKC, Ferrari I, et al. Distribution of CGG repeats and FRAXAC1/DXS548 alleles in South American populations. *Am J Med Genet.* (2002) 111:243–52. doi: 10.1002/ajmg.10572
28. Chiurazzi P, Destro-Bisol G, Genuardi M, Oostra BA, Spedini G, Neri G. Extended gene diversity at the FMR1 locus and neighbouring CA repeats in a sub-Saharan population. *Am J Med Genet.* (1996) 64:216–9. doi: 10.1002/(SICI)1096-8628(19960712)64:1<216::AID-AJMG39>3.0.CO;2-O
29. Jara L, Aspillaga M, Avendaño I, Obreque V, Blanco R, Valenzuela CY. Distribution of (CGG)n and FMR-1 associated microsatellite alleles in a normal Chilean population. *Am J Med Genet.* (1998) 75:277–82. doi: 10.1002/(SICI)1096-8628(19980123)75:3<277::AID-AJMG10>3.0.CO;2-M
30. Zhou Y, Tang K, Law H-Y, Ng ISL, Lee CGL, Chong SS. FMR1 CGG repeat patterns and flanking haplotypes in three Asian populations and their relationship with repeat instability. *Ann Hum Genet.* (2006) 70:784–96. doi: 10.1111/j.1469-1809.2006.00265.x
31. Crawford DC, Schwartz CE, Meadows KL, Newman JL, Taft LF, Gunter C, et al. Survey of the fragile X syndrome CGG repeat and the short-tandem-repeat and single-nucleotide-polymorphism haplotypes in an African American population. *Am J Hum Genet.* (2000) 66:480–93. doi: 10.1086/302762
32. Barros-Núñez P, Rosales-Reynoso MA, Sandoval L, Romero-Espinoza P, Troyo-Sanromán R, Ibarra B. Genetic variation of the FMR1 gene among four Mexican populations: Mestizo, Huichol, Purepecha, and Tarahumara. *Am J Hum Biol Off J Hum Biol Counc.* (2008) 20:259–63. doi: 10.1002/ajhb.20705
33. Fernandez-Carvajal I, Walichiewicz P, Xiaosen X, Pan R, Hagerman PJ, Tassone F. Screening for Expanded Alleles of the FMR1 Gene in Blood Spots from Newborn Males in a Spanish Population. *J Mol Diagn JMD.* (2009) 11:324–9. doi: 10.2353/jmoldx.2009.080173
34. Rosales-Reynoso MA, Mendoza-Carrera F, Troyo-Sanromán R, Medina C, Barros-Núñez P. Genetic diversity at the FMR1 locus in Mexican population. *Arch Med Res.* (2005) 36:412–7. doi: 10.1016/j.arcmed.2004.05.005
35. Biancalana V, Glaeser D, McQuaid S, Steinbach P, EMQN. best practice guidelines for the molecular genetic testing and reporting of fragile X syndrome and other fragile X-associated disorders. *Eur J Human Genet.* (2015) 23:417–25. doi: 10.1038/ejhg.2014.185

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Seizures in Fragile X Syndrome: Associations and Longitudinal Analysis of a Large Clinic-Based Cohort

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Fragile X syndrome (FXS), the most common inherited cause of intellectual disability, learning disability, and autism spectrum disorder, is associated with an increased prevalence of certain medical conditions including seizures. The goal of this study was to better understand seizures in individuals with FXS using the Fragile X Online Registry with Accessible Research Database, a multisite observational study initiated in 2012 involving FXS clinics in the Fragile X Clinic and Research Consortium. Seizure data were available for 1,607 participants, mostly male (77%) and white (74.5%). The overall prevalence of at least one seizure was 12%, with this rate being significantly higher in males than females (13.7 vs. 6.2%, $p < 0.001$). As compared to individuals with FXS without seizures, those with seizures were more likely to have autism spectrum disorder, current sleep apnea, later acquisition of expressive language, more severe intellectual disability, hyperactivity, irritability, and stereotyped movements. The mean age of seizure onset was 6.4 (SD 6.1) years of age with the great majority (>80%) having onset of seizures which was before 10. For those with epilepsy, about half (52%) had seizures for more than 3 years. This group was found to have greater cognitive and language impairment, but not behavioral disruptions, compared with those with seizures for < 3 years. Antiepileptic drugs were more often used in males (60.6%) than females (34.8%), and females more often required more than one medication. The most commonly used anticonvulsants were oxcarbazepine, valproic acid, lamotrigine, and levetiracetam. The current study is the largest and first longitudinal study ever conducted to describe seizures in FXS. Overall, this study confirms previous reports of seizures in FXS and extends previous findings by further defining the cognitive and behavioral phenotype of those with epilepsy in FXS. Future studies should further investigate the natural history of seizures in FXS and the characteristics of seizures in FXS in adulthood.

Keywords: Fragile X syndrome, seizures, epilepsy, longitudinal, autism spectrum disorder

INTRODUCTION

Fragile X syndrome (FXS) is the most common known inherited cause of intellectual disability, learning disability, and autism spectrum disorder (ASD), with an estimated prevalence of about 1/4,000 (1). Individuals with FXS display variable physical features such as large ears, long face, macrocephaly, macroorchidism, and variable levels of functioning, with a fairly stereotyped cognitive profile and behavioral features characterized by hyperactivity, anxiety, and socialization difficulties. Additionally, certain medical problems are more common in FXS, such as strabismus, otitis media, gastroesophageal reflux, loose stools, sleep apnea, and seizures (2).

FXS results from a trinucleotide repeat (CGG) expansion mutation of >200 repeats (full mutation) in the promoter of *FMR1* (*fragile X mental retardation 1*) gene (3) which leads to transcriptional silencing of *FMR1* and loss or significant reduction of expression of the gene product, FMRP (fragile X mental retardation protein) (4). FMRP is an RNA-binding protein that appears to function as a dendritic translational repressor that modulates receptor-activated dendritic protein synthesis and regulates multiple ion channels (5–7). In the absence of FMRP in the *Fmr1* knockout (k/o) mouse model of FXS, there is immature dendritic spine morphology and region- and cell-dependent deficits in synaptic plasticity (8). Accordingly, these synaptic abnormalities result in abnormal epileptiform discharges (9) and a high frequency of audiogenic seizures in the *Fmr1* k/o mouse (10), presumably modeling the increased risk of seizures in humans with FXS.

Past reports have identified seizures in 4.4%-40% of males with FXS (2, 11–19) with a lower frequency of 4.4%-18% in larger cohorts with less referral bias (2, 13, 15–19). Many children with FXS also have abnormal electroencephalograms (EEGs) without overt epileptic seizures (15, 17, 19), frequently with a pattern of centrotemporal spikes (similar to benign focal epilepsy of childhood) (13, 15, 19, 20). Types of seizures reported in FXS vary widely, and clinical and EEG findings consistent with Panayiotopoulos syndrome, a benign autonomic epilepsy syndrome, have also been seen in FXS (21). Complex partial (focal onset with impaired awareness) seizures were reported as most common in FXS in several series (13, 15, 18), although simple partial (focal onset without impaired awareness) and generalized tonic-clonic seizures occur often, and status epilepticus has also been observed (22). Seizures are reported to be easily controlled in most cases (15, 17, 18) and resolve during childhood in the majority of individuals with FXS, although in a study of hospital encounters for adolescents and adults with FXS, seizures represented an identifiable reason for emergency room presentation and hospitalization in FXS (23). Occasional patients with FXS and intractable seizures due to mesial temporal sclerosis have been described, suggesting a secondary etiology amenable to surgical management (24). A small case series describing three females with FXS and severe developmental impairment and medically refractory focal epilepsy also suggests that severe epilepsy in FXS may signal a secondary genetic condition (25).

Studies investigating the association of seizures with other disease features in FXS include a small cohort which showed a trend toward an increased rate of seizures in individuals who were also diagnosed with ASD (26) and a second small case series of 11 patients which suggested an association between epilepsy and attention problems (27). In the largest investigation of seizures in FXS involving 1,394 individuals assessed by a survey and 352 individuals assessed in clinic (19), 14% of males and 6% of females reported seizures. Seizures tended to be infrequent and more often focal, had onset between 4 and 10 years of age, were generally easily treated, and were associated with ASD, but not academic achievement. A clinic-based study of 135 patients with FXS seen in clinic (17) suggested that many patients described spells such as staring, but most of these were without EEG correlate, and only 4.4% of the cohort were actually diagnosed with seizures, emphasizing the importance of clinical confirmation of seizure diagnoses in persons with FXS. A large study of ASD in FXS based on data from 547 participants with FXS in the Fragile X Online Registry and Accessible Research Database (FORWARD) natural history study (28) showed that having a diagnosis of ASD was associated with an approximately three-fold increased risk of seizures both in younger children and in adolescent/young adults (29).

The Fragile X Clinic and Research Consortium (FXCRC) initial database collected data from 260 participants with FXS from 9 FXS Clinics from 2005 to 2011 and showed a seizure frequency of 10% (12% in males) (2). Longitudinal data about seizures from participants with FXS have subsequently been collected as part of the FORWARD project (28), from 2012 to 2021. In the present report, we use these FORWARD data to investigate seizure prevalence and characteristics, including gender differences, age of onset and resolution, duration and associated comorbid conditions, and other features (e.g., use of anticonvulsants), in the largest and first longitudinal study ever conducted to describe seizures in FXS. Specific goals of these analyses are to update information on seizure frequency and co-occurring problems associated with seizures in FXS in a larger group than previously reported, study the onset and trajectory of seizures experienced by those with FXS across time with longitudinal data, and understand factors associated with more severe epilepsy in FXS.

MATERIALS AND METHODS

Data analyzed for this report were derived from FORWARD. As described previously (28), FORWARD is a multisite observational study initiated in 2012. The study collects data yearly on a Registry form, Parent Report form, and Clinician Report form, as well as standardized questionnaires including the Aberrant Behavior Checklist-Community Edition (ABC-C) (29), the Social Communication Questionnaire (SCQ) (30), and the Social Responsiveness Scale – Second Edition (SRS-2) (31). The analyses for this report were performed using baseline and longitudinal data collected during follow-up visits from FORWARD Version 5, with data obtained from 1,607 individuals with FXS evaluated between 2012 and 2020, who had

information available about seizures derived from the Clinician Report form. There were 1,607 baseline visits and 1,945 follow-up visits (occurring after the baseline visit) for 803 participants with at least 1 year of follow-up. The study was approved by the Institutional Review Board for each participating FXS Clinic where data were collected, and written informed consent was obtained from primary caregivers or adult patients who were their own guardians.

Demographic variables including age, sex, and ethnicity were collected on the Registry form. Data from the Clinician Report form included variables related to seizures including presence or absence of seizures currently or in the past, age (in years and months) at seizure onset, age when seizures resolved if the participant had been 2 years without seizures, type of seizures (focal, generalized, febrile), whether antiepileptic medication was used for seizures at the time of the visit, and, if so, which antiepileptic medication(s) the participant was taking. Antiepileptic medication use was tracked at all visits and was reported in this paper based on use described at any visit over the course of follow-up. Other data from the Clinician Report form about problems that could potentially be associated with seizures were also utilized, including level of intellectual disability (ID), presence of ASD, presence of sleep apnea, severity of behaviors on the ABC-C adjusted for FXS (ABC_{FX}) (32), and severity of ASD symptoms on the SCQ and SRS-2.

STATISTICAL ANALYSIS

Frequency tabulations and proportions for categorical variables, and means and standard deviations for continuous variables, were used for the descriptive analyses. The chi-square test or Fisher's exact test for categorical variables, and analysis of variance (ANOVA) for continuous variables were used to compare characteristics of those with and without seizure experience and to compare males and females with seizures. Using longitudinal data, subjects were divided into three groups based on seizure experience. Participants who reported no seizures ever and who were observed at least once at or over the age of 15 comprised one group; participants who reported having seizures but only for a period <3 years and were followed at least 2 years after the last reported seizure comprised a second group; and participants who reported seizures over a period >3 years comprised the third group. Comparisons of demographic and clinical variables between these three groups were made using chi-square tests or Fisher's exact tests, for categorical variables, and Wilcoxon rank-sum test and Student's *t*-tests for continuous variables, as appropriate.

Kaplan–Meier survival estimates were produced to model and plot time to first reported seizure for the full sample. Mixed-effect logistic regression models were fit to estimate the rate of current seizures for age groups, using the longitudinal data, across the full sample. This model featured random intercepts for subject.

Analyses were performed using SPSS version 26 and SAS version 9.4. In each analysis reported in this paper, data were used for all individuals who had valid values for the variables used in that analysis. Statistical significance level was set at 5%.

TABLE 1 | Demographics of the FORWARD cohort.

Characteristic (*N*)*	
Sex ($N = 1607$)	77% male
Race/ethnicity ($N = 1607$)	74.5% White, 7.7% African American, 3.4% Asian, 12.9% Hispanic, 1.4% Other
Age at first visit (years, $N = 1607$)	26.3% age 0–5, 27.1% age 6–10, 21.0% age 11–15, 11.4% age 16–20, 14.2% age 21+
Number of visits ($N = 1607$)	1–804, 2–329, 3–191, 4–103, 5–73, 6–39, 7–39, 8–28, 9–1
ASD by clinician (DSM5, $N = 1592$)	39.5%
ASD by SCQ ($N = 1187$)	61% no ASD, 39% ASD
ASD by SRS ($N = 1061$)	16% no ASD, 42% mild ASD, 42% severe ASD
Intellectual disability ($N = 1536$)	7.7% normal, 15.4% DD, 7.4% borderline, 23.2% mild, 38.7% mod, 7.2% severe, 0.3% profound
Current or past seizures ($N = 1607$)	12%
Current or past sleep apnea ($N = 1323$)	16.9%

**N is less than total based on missing data for some items, and the SCQ and SRS were filled out separately from the Clinician forms and not collected on all individuals.*

RESULTS

Characteristics of the 1,607 FORWARD participants with available seizure data used for this study are shown in **Table 1**: 77% were male, 74.5% were White, 7.7% were African American/Black, 3.4% were Asian, and 12.9% were Hispanic. The mean age at the baseline evaluation for the cohort was 13.8 years. A history of current or past seizures at any evaluation was reported for 193 patients (170 males and 23 females) or 12% of the sample. Approximately half had moderate (38.7%) or severe/profound (7.5%) ID, ∼39.5% were diagnosed with ASD by a clinician, and sleep apnea was present in 16.9% of the cohort. Severity of ID, ASD symptom severity by SRS-2, and percent of patients with an ASD diagnosis by SCQ were all lower in females with FXS relative to males.

In the entire cohort, there were a higher proportion of males with FXS relative to females who had current or past seizures at any time prior to baseline or during follow-up, 13.7 and 6.2%, respectively (Fisher's exact $p < 0.0001$). Seizures occurred more often in severely affected patients, with 68.7% of patients with seizures having moderate, severe, or profound ID, while only 43% of patients without seizures had this level of ID (chi-square $p < 0.0001$ for distribution of ID within the seizure and no seizure groups, **Table 2**, **Figure 1A**). Those with seizures had later onset of spoken language (age 2.37 vs. 2.02 years, ANOVA $p = 0.03$). Those with seizures were more likely to have an ASD diagnosis compared to those without seizures (53.9 vs. 41.9%, chi-square $p < 0.0001$, **Table 2**, **Figure 1B**). The group with seizures had a trend to have an increased likelihood of a diagnosis of ASD by SCQ (score of over 15) at the baseline FORWARD visit (45.9 vs. 38.3%, Fisher's exact $p = 0.087$), although not statistically significant, and increased severity of

TABLE 2 | Association of cohort characteristics with seizures.

Characteristic	Seizures (*N* = 193)	No seizures (*N* = 1,414)	*p*-value**
	N (% of total with seizures or no seizures)		
Sex			<0.0001
Male	170 (88.1)	1067 (75.4)	
Female	23 (11.9)	347 (24.6)	
Age at first visit*	13.7 (8.7)	11.6 (9.4)	0.004
Age at last visit*	16.1 (8.9)	13.5 (9.8)	<0.00001
Race/ethnicity			
White non-Hispanic	139 (72.0)	1061 (75.0)	0.6
Black non-Hispanic	17 (8.8)	106 (7.5)	
Asian	9 (4.7)	45 (3.2)	
Hispanic	24 (12.4)	184 (13.0)	
Other	4 (2.1)	18 (1.3)	
ASD diagnosis			<0.00001
No	80 (41.9)	847 (60.5)	
Yes	103 (53.9)	526 (37.5)	
ASD by SCQ			0.087
No	80 (54.1)	641 (61.7)	
Yes	68 (45.9)	398 (38.3)	
Unknown	8 (4.2)	28 (2)	
ASD by SRS-2			0.008
Absence of ASD	12 (9.2)	153 (16.4)	
Mild ASD	48 (36.9)	401 (43.1)	
Severe ASD	70 (48)	377 (40.5)	
Level of ID			<0.00001
None	5 (2.7)	114 (3.0)	
Devel Delay	11 (5.9)	226 (16.7)	
Borderline	7 (3.8)	107 (7.9)	
Mild	35 (18.8)	322 (23.9)	
Moderate	102 (54.8)	493 (36.5)	
Severe	25 (13.4)	85 (6.3)	
Profound	1 (0.5)	3 (0.2)	
Sleep apnea			0.001
Currently	7 (4.2)	29 (2.5)	
Past	7 (4.2)	69 (6.0)	
No	130 (78.8)	1000 (86.4)	
Don't know	21 (12.7)	60 (5.2)	

**Values reported are mean (SD). **Significance (p-value) is reported for distribution of categories (chi-squared) in characteristic and indicates whether these are differently distributed between the group with seizures and those without.*

ASD symptoms by SRS-2 (9.2 vs. 16.4% absence of ASD, 53.8 vs. 40.5% severe ASD, chi-square $p = 0.008$). Current symptoms of obstructive sleep apnea were seen more often in those with seizures (4.2 vs. 2.5%) which led to a chi-square of $p = 0.001$ for the distribution of sleep apnea category between those with and without seizures. However, if one considered past history of sleep apnea, the distribution of seizures was similar in those with and without sleep apnea, making it unclear whether there is a true association. There were no effects of race/ethnicity, place of residence, household income, or level of education of parents on the likelihood of patients manifesting seizures at any time.

Patients were more likely to have reported seizures if they were older at the first and last evaluations (chi-square $p < 0.0001$ for age distribution between those with and without seizures), thought likely to be related to being past the typical age of onset of seizures in FXS. There was no effect of the number of evaluations in FORWARD.

In the group of 193 patients with seizures (**Table 3**), the mean age at the baseline visit was 13.7 ± 8.7 and the number of evaluations during follow-up was 2.5 ± 1.8. The mean age of seizure onset was 6.4 ± 6.1, and the mean time elapsed since the last seizure was 6.4 ± 7.7 years. In most cases, seizure onset occurred before the age of 10, including 86.7 and 81.8% of males and females, respectively (**Figure 2A**). For males ($N = 170$), 96% had the first seizure by age 15, with only 4% presenting after age 15. For females ($N = 23$), 18.2% had their first seizure after age 15, a trend in age distribution with borderline significance ($p = 0.058$). The age of the last seizure followed a similar distribution to age of onset of seizures with 70.9% and 63.6% of seizures resolving by age 10 in males and females, respectively (**Figure 2B**). Only 7.9% of males and 18.2% of females had seizures continuing after age 20; this sex difference, based on a low number of females, was not significant.

Partial seizures were reported in 25% and generalized seizures in 31% of patients, with febrile seizures in 8% and the remainder of seizures being of unknown type (**Figure 3A**). Males and females did not show a different distribution of seizure types.

The average number of anticonvulsants used across the entire group with seizures was 0.91 ± 1.01. Anticonvulsant use was reported in 60.6% of males with seizures at any evaluation vs. 34.8% of females (Fisher's exact $p = 0.024$, **Figure 3B**), with 37.6% of males vs. 13.0% of females requiring a single medication (chi-square $p = 0.035$), and 23 and 22%, respectively, requiring two or more medications. The most commonly used anticonvulsant was oxcarbazepine, followed by valproic acid, lamotrigine, and levetiracetam (**Figure 3C**).

During the follow-up period, 26 incident seizures were reported in 20 males and 6 females in 1945 patient-years of follow-up, for a calculated rate of 0.013 new seizures per patient year of follow-up. Incident seizures had an age and sex distribution resembling that for age and sex distribution of seizures at baseline. Twelve males experienced a change in seizure type in 283 patient years of follow-up, indicating that seizure type changes about 4.2% of time in follow-up. **Figure 4A** shows a Kaplan–Meier plot of age of seizure onset in the FXS cohort, which reached a plateau by age 15 years. There was also an apparent increase in seizure onset after age 25, despite the low actual numbers of adults with seizures. This is most likely because the Kaplan–Meier model estimated the proportion based only on the subjects observed until later ages, even though many of the younger subjects would have likely reached age 25 years without having a seizure. For that reason, the Kaplan–Meier estimate of the total proportion experiencing seizures (about 17%) is greater than the raw calculation of the proportion reporting seizures (12% as noted above). **Figure 4B** shows the proportion of patients having seizures separated into 5 year groups. As expected, the proportions are higher in the youngest groups, tapering off until reaching the 26–30 year age group.

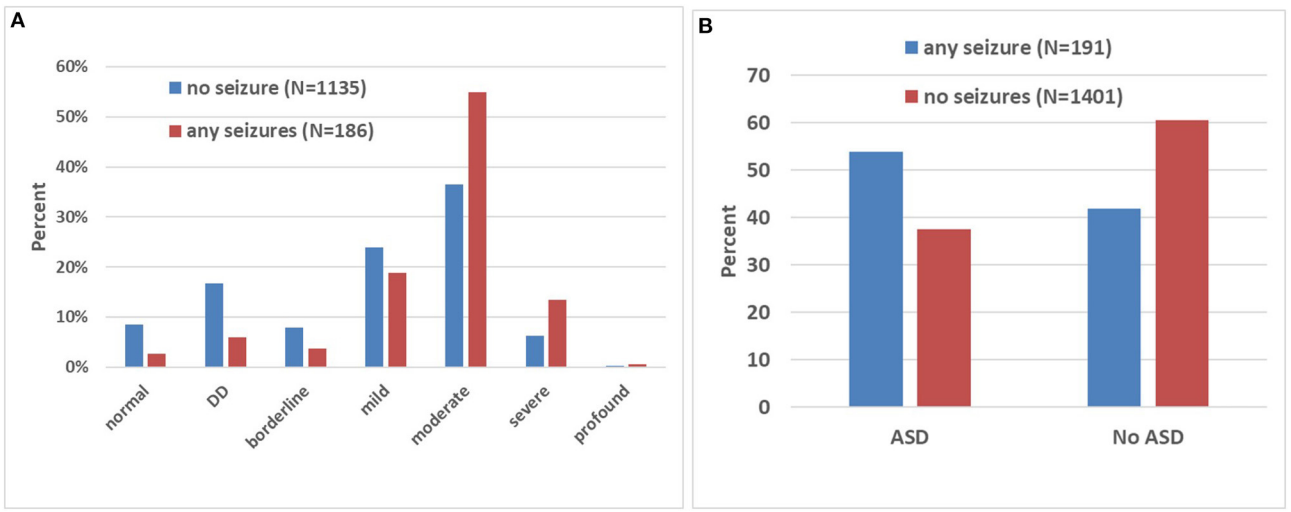

FIGURE 1 | Association between intellectual disability (ID), autism spectrum disorder (ASD), and seizures in FXS. **(A)** The bar graph depicts the proportion (in percentages) of individuals with and without seizures at each level of ID. Note the higher proportion of patients with seizures at the higher levels of ID severity. DD corresponds to developmental delay in younger individuals. **(B)** The graph shows the proportion (in percentages) of individuals with and without seizures diagnosed (or not) with ASD. Note in the group with ASD, a higher proportion of patients with seizures.

TABLE 3 | Characteristics of the FXS FORWARD participants with seizures.

Sex		# of evaluations (clinician form)	Age at first evaluation	Age of seizure onset	SCQ Total Score	Total SRS T Scores	# of unique medications reported across all visits	Years from last seizure onset to last evaluation
1 Male	Mean	2.48	13.39	6.22	16.17	76.12	0.95	6.42
	N	170	170	150	131	114	170	151
	SD	1.860	8.748	5.690	6.450	11.161	1.010	7.647
2 Female	Mean	2.35	15.83	7.36	11.94*	75.69	0.61	6.23
	N	23	23	22	17	16	23	22
	SD	1.402	8.072	8.255	4.905	13.489	0.941	7.994
Total	Mean	2.47	13.68	6.37	15.68	76.07	0.91	6.39
	N	193	193	172	148	130	193	173
	SD	1.809	8.686	6.060	6.422	11.415	1.006	7.668

*$p = 0.01$, no other characteristics are significantly different between males and females.

Patients with persistent seizures, operationally defined as those with seizures lasting over 3 years ($N = 75$), were compared with those with seizures lasting <3 years ($N = 70$) and those without seizures who had reached age 15 ($N = 514$) (**Table 4**). The group without seizures was limited to those over 15 years to ensure that the comparator group had a very low likelihood of developing seizures. Those without seizures were older at the first and last evaluation than the other groups; however, this was expected given the age limitation on this group. There was no significant difference in age at evaluation between the two seizure groups. Relative to those with seizures <3 years, those with seizures lasting >3 years were on more anticonvulsants ($p < 0.0001$ both first and last evaluations), were more likely to have partial seizures and less likely to have febrile seizures ($p = 0.005$ at first evaluation and 0.03 at last evaluation), made less language progress during follow-up, had more severe language impairment at the most recent visit ($p = 0.03$), and had a higher proportion of severe/profound ID (although this was not statistically significant, $p = 0.14$), but did not have higher prevalence of ASD, sleep apnea, or severity of behavioral issues on any subscale of the ABC_{FX}. The lack of language progress in the group with seizures lasting >3 years was remarkable, with 25% non-verbal at the initial visit and 21.4% remaining non-verbal at the last visit compared to 14.4% non-verbal at the initial visit and 5.7% at the last visit for those with seizures lasting <3 years. Underscoring the phenotypical severity associated with having seizures regardless of duration, those with no seizures had significantly less severe ID than the <3 year seizure group ($p = 0.003$), less severe language impairment at the first but not the last visit for those with seizures <3 years ($p = 0.038$ and

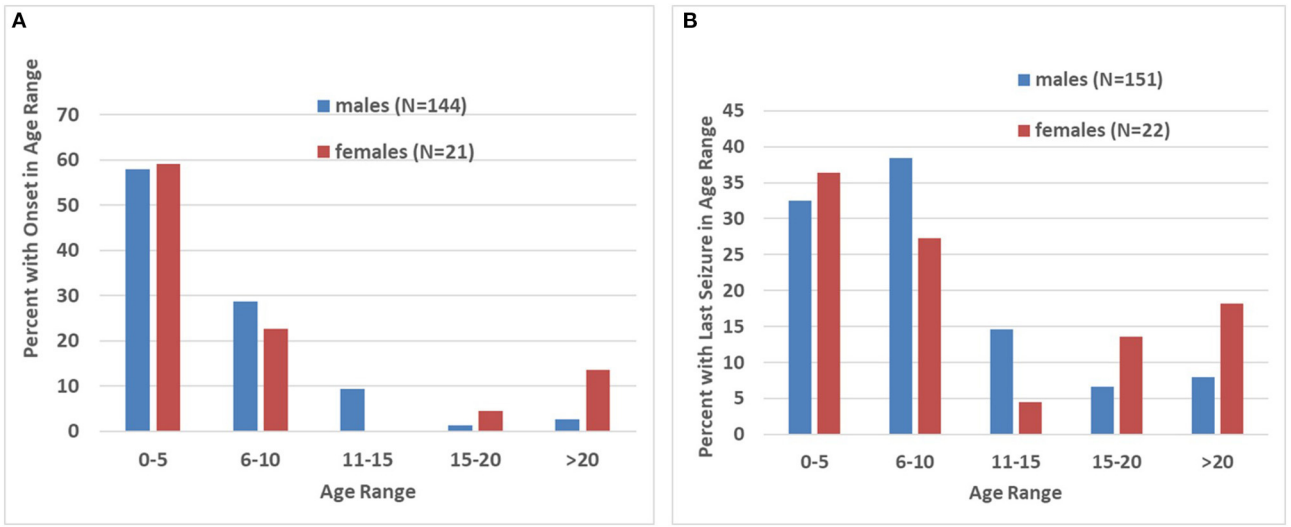

FIGURE 2 | Age of onset and resolution of seizures. **(A)** Bar graph showing the proportion (in percentages) of individuals, divided by sex, with onset of seizures at different 5 year bins. Note that, with exception of a subset of females with onset after age 15, most patients with FXS displayed an onset before age 10. **(B)** Bar graph showing the proportion (in percentages) of individuals, divided by sex, experiencing last seizures at different 5 year bins. As for seizure onset, most patients had their last seizure before age 15, with exception of a subset of females who has seizure resolution after this age.

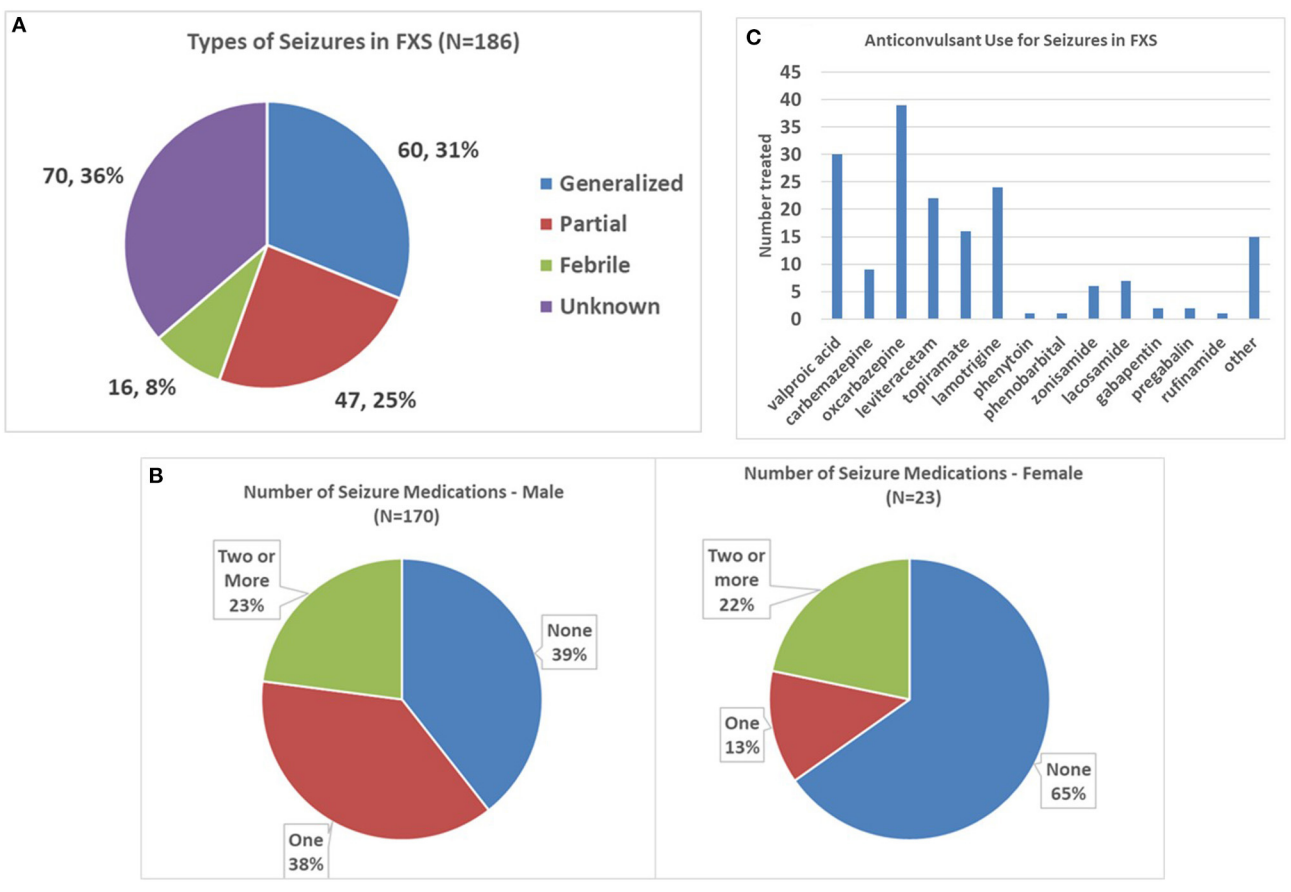

FIGURE 3 | Seizure types and anticonvulsant use in FXS. **(A)** Pie graph depicting the proportion of each seizure type in the entire FXS cohort. **(B)** Graphs showing the number of anticonvulsants per patient in males and females, respectively. Note the higher proportion of males on anticonvulsants. **(C)** Number of patients with FXS using the most common anticonvulsants. Oxcarbazepine, valproic acid, and lamotrigine were the three most commonly prescribed anticonvulsants.

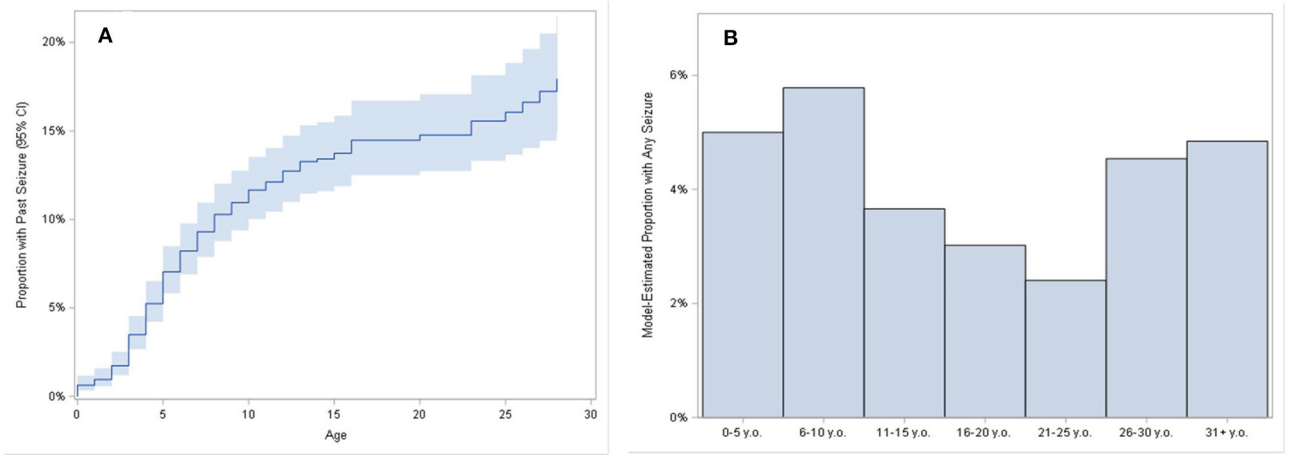

FIGURE 4 | Plot of time to first seizure. **(A)** Reverse Kaplan–Meier estimates show the estimated proportion of subjects who had their first seizure onset reported, over ages 0 to 30. Band shows 95% confidence intervals for estimates. For example, approximately 7% of subjects had first seizure onset by age 5, approximately 12% of subjects had first seizure onset by age 10, and approximately 14% of subjects had first seizure onset by age 15. Bar chart of the model-estimated proportion of having any seizures during selected age periods **(B)**. Proportion estimates are derived from mixed-effect logistic regression models fit for the odds of having experienced a seizure, with categorized age as the predictor, and random intercepts for subject.

0.15, respectively), less severe language impairment than those with seizures >3 years at both visits ($p = 0.0004$ and 0.0062, respectively), a higher percent with sustained conversation ability at both visits than those with seizures <3 years at both visits ($p = 0.0003$ and 0.01, respectively), a lower proportion with ASD diagnosis than both seizure groups at both visits ($p = 0.009$ for both groups at the last visit), and lower scores on the ABC_{FX} Hyperactivity, Irritability, and Stereotypy subscales than both seizure groups at both visits (p-values ranging from <0.0001 to 0.029).

DISCUSSION

In this study, we characterized seizures in the largest clinically evaluated cohort of individuals with FXS. In addition, this is the only longitudinal study of seizures to date in FXS and thus the only study ever to evaluate incident onset of seizures prospectively in this syndrome. In addition to confirming previously reported characteristics of individuals with FXS and seizures, such as prevalence, sex distribution (13.7% males, 6.2% females), and association with ASD (19, 26, 33), we identified an association with current sleep apnea and additional features. Those with seizures were also more likely to have more severe ID and later onset of expressive language, findings which have not been reported before, likely due to lack of sufficient numbers of subjects (10–15, 26) or lack of clinical assessment or evaluation of levels of ID and language (19, 33) in prior studies. The group with seizures was also more likely to have more severe behavioral problems, as measured by the ABC_{FX} (Hyperactivity, Irritability, and Stereotypy subscales). Availability of longitudinal data allowed the delineation of key aspects in the evolution of seizures in FXS and their association with other features of the disorder. A longer course of epilepsy in FXS was associated with greater overall cognitive and language impairments, but not more severe behavioral problems.

In this FORWARD cohort, individuals with FXS and seizures were more likely to have ASD diagnosed by the clinician based on DSM5 criteria. The association between seizures and a clinical diagnosis of ASD in children with FXS is supported by the finding that this subset has more severe ASD symptoms on the SCQ and SRS-2. Consistent with our findings, previous studies have found an association between the diagnosis of ASD and seizures and children with ASD with seizures have been found to have more severe ASD symptoms (34, 35). However, although previous studies suggested that children with ASD and genetic disorders have high rates of epilepsy (36), this study suggests that the rate of seizures in FXS is rather moderate, at 12%, comparable to the 12.1% rate of epilepsy in individuals with idiopathic ASD reported in a recent meta-analysis (37). Additionally, studies on children with ASD also demonstrate that many have subclinical epileptiform discharges in a centrotemporal pattern (38, 39) and occipital regions (40), similar to EEG profiles reported in FXS (13, 15, 19–21). Children with ASD and EEGs with subclinical epileptiform discharges are found to be more severely impaired (35, 41) but may show improvement in behavior and cognition with treatments targeted to reduced epileptiform discharges (35, 38, 42). Although EEG data were not collected in the FORWARD study, children with FXS and an abnormal EEG are a subgroup of FXS patients that may warrant closer study.

Onset of seizures in FXS was typically (>80%) below age 10, with a very low proportion of males having seizure onset after age 15 years. A higher percent of females had seizure onset after age 15 years, which suggests a slightly different pattern for seizure onset in females, although females with first seizure at age >15 represented only four patients. Thus, the marginally significant chi-square ($p = 0.058$) for a different male–female distribution of age of seizure onset may be due to the relatively small number

TABLE 4 | Comparison of characteristics between groups with no seizures, seizures reported for < 3 years, and seizures reported for > 3 years*.

	Overall (*N* = 659)		No Seizures (*N* = 514)		Seizures < 3 years (*N* = 70)		Seizures > 3 years (*N* = 75)		*p*-values			
Variable	***n***	**% or Mean (SD)**	***n***	**% or Mean (SD)**	***n***	**% or Mean (SD)**	***n***	**% or Mean (SD)**	**Overall**	**1 vs. 2**	**1 vs. 3**	**2 vs. 3**
Number of evaluations	659	2.8 (2.0)	514	2.8 (2.0)	70	2.6 (1.7)	75	2.8 (2.1)	0.7533	0.4488	0.8982	0.6178
Number of evaluations [Min, Max]	659	(1, 9)	514	(1, 9)	70	(1, 8)	75	(1, 8)				
Number of evaluations–binary									0.5789	0.8537	0.3185	0.3821
1	235	35.7%	180	35.0%	24	34.3%	31	41.3%				
2+	424	64.3%	334	65.0%	46	65.7%	44	58.7%				
Gender									0.0535	0.0537	0.1033	0.7522
Male	520	79.0%	395	77.0%	61	87.1%	64	85.3%				
Female	138	21.0%	118	23.0%	9	12.9%	11	14.7%				
Age at first evaluation	659	19.9 (9.6)	514	21.1 (9.4)	70	14.7 (8.3)	75	16.4 (9.5)	<0.0001	<0.0001	<0.0001	0.2669
Age at last evaluation	659	22.6 (9.3)	514	23.8 (9.0)	70	17.6 (8.4)	75	18.9 (9.6)	<0.0001	<0.0001	<0.0001	0.3685
Years between first and last evaluation	419	4.3 (2.2)	330	4.3 (2.3)	46	4.3 (2.1)	43	4.4 (2.2)	0.9798	0.8835	0.4309	0.4973
Age of onset	135	6.7 (6.6)		N/A	66	5.9 (5.3)	69	7.5 (7.6)	N/A	N/A	N/A	0.1625
Mean inter-evaluation period	423	1.8 (1.2)	333	1.8 (1.2)	46	2.0 (1.2)	44	1.6 (1.0)	0.3655	0.3427	0.3576	0.1326
Number of AEDs first evaluation	659	0.1 (0.5)	514	0.0 (0.0)	70	0.3 (0.5)	75	1.1 (1.0)	<0.0001	<0.0001	<0.0001	<0.0001
Number of AEDs first evaluation [Median (IQR)]	659	0.0 (0.0-0.0)	514	0.0 (0.0-0.0)	70	0.0 (0.0-0.0)	75	1.0 (0.0-2.0)				
Number of AEDs last evaluation	659	0.2 (0.5)	514	0.0 (0.0)	70	0.3 (0.5)	75	1.3 (1.0)	<0.0001	<0.0001	<0.0001	<0.0001
Number of AEDs last evaluation [Median (IQR)]	659	0.0 (0.0-0.0)	514	0.0 (0.0-0.0)	70	0.0 (0.0-0.0)	75	1.0 (1.0-2.0)				
Seizure type first seizure evaluation									N/A	N/A	N/A	0.0051
Generalized	61	45.5%		N/A	28	43.8%	33	47.1%				
Partial	46	34.3%		N/A	16	25.0%	30	42.9%				
Febrile	11	8.2%		N/A	10	15.6%	1	1.4%				
Unknown	16	11.9%		N/A	10	15.6%	6	8.6%				
Seizure type last seizure evaluation									N/A	N/A	N/A	0.0363
Generalized	39	45.9%		N/A	19	42.2%	20	50.0%				
Partial	25	29.4%		N/A	10	22.2%	15	37.5%				
Febrile	11	12.9%		N/A	10	22.2%	1	2.5%				
Unknown	10	11.8%		N/A	6	13.3%	4	10.0%				
Intellectual disability Dx last evaluation									0.0001	0.0003	0.0735	0.1380
No ID	35	5.5%	31	6.3%	4	5.8%	0	0.0%				
Delayed development	3	0.5%	0	0.0%	3	4.3%	0	0.0%				
Borderline	35	5.5%	30	6.1%	2	2.9%	3	4.3%				
Mild	146	23.0%	119	24.0%	11	15.9%	16	22.9%				
Moderate	356	56.2%	274	55.4%	42	60.9%	40	57.1%				
Severe	57	9.0%	40	8.1%	7	10.1%	10	14.3%				
Profound	2	0.3%	1	0.2%	0	0.0%	1	1.4%				
Language milestone (Q24) first evaluation									0.0049	0.0378	0.0004	0.6416
Non-verbal	12	1.8%	6	1.2%	2	2.9%	4	5.3%				
Signing	7	1.1%	5	1.0%	0	0.0%	2	2.7%				
Babbling	2	0.3%	0	0.0%	1	1.4%	1	1.3%				
Few words	52	8.0%	34	6.7%	7	10.1%	11	14.7%				
Word combinations	64	9.9%	47	9.3%	9	13.0%	8	10.7%				
Phrases	512	78.9%	413	81.8%	50	72.5%	49	65.3%				

(Continued)

TABLE 4 | Continued

Variable	Overall (*N* = 659)		No Seizures (*N* = 514)		Seizures < 3 years (*N* = 70)		Seizures > 3 years (*N* = 75)		*p*-values			
	n	% or Mean (SD)	*n*	% or Mean (SD)	*n*	% or Mean (SD)	*n*	% or Mean (SD)	Overall	1 vs. 2	1 vs. 3	2 vs. 3
Sustained conversation (Q26) first evaluation									0.0009	0.0774	0.0003	0.5314
Non-verbal	26	4.1%	15	3.0%	4	6.0%	7	9.6%				
Yes, sustained conversion	385	60.3%	320	64.1%	34	50.7%	31	42.5%				
No sustained conversion	228	35.7%	164	32.9%	29	43.3%	35	47.9%				
Language milestone (Q24) last evaluation									0.0034	0.1536	0.0062	0.0358
Non-verbal	8	1.2%	5	1.0%	1	1.4%	2	2.7%				
Signing	8	1.2%	5	1.0%	2	2.9%	1	1.3%				
Babbling	4	0.6%	2	0.4%	0	0.0%	2	2.7%				
Few words	40	6.1%	28	5.5%	1	1.4%	11	14.7%				
Word combinations	53	8.1%	37	7.3%	10	14.3%	6	8.0%				
Phrases	538	82.6%	429	84.8%	56	80.0%	53	70.7%				
Sustained conversation (Q26) last evaluation									0.0236	0.2432	0.0122	0.1287
Non-verbal	12	2.0%	9	1.9%	0	0.0%	3	4.5%				
Yes, sustained conversion	404	65.8%	329	68.4%	41	62.1%	34	50.7%				
No sustained conversion	198	32.2%	143	29.7%	25	37.9%	30	44.8%				
Autism Dx ever									0.0003	0.0005	0.0246	0.1952
No	321	48.9%	273	53.2%	20	28.6%	28	37.8%				
Yes	328	49.9%	234	45.6%	48	68.6%	46	62.2%				
Autism Dx current (last evaluation)									0.0022	0.0095	0.0094	0.3945
Yes	276	43.0%	196	39.0%	39	57.4%	41	57.7%				
No	331	51.6%	277	55.1%	28	41.2%	26	36.6%				
Don't know	35	5.5%	30	6.0%	1	1.5%	4	5.6%				
Sleep apnea last evaluation									0.1434	0.3075	0.0555	0.6080
No	378	97.2%	295	98.0%	43	95.6%	40	93.0%				
Yes	11	2.8%	6	2.0%	2	4.4%	3	7.0%				
ABC hyperactivity first evaluation	506	18.8 (7.4)	393	17.9 (6.9)	58	23.3 (8.1)	55	20.6 (8.0)	<0.0001	<0.0001	0.0076	0.0795
ABC inappropriate Speech first evaluation	506	8.4 (3.4)	393	8.3 (3.4)	58	9.1 (3.6)	55	8.1 (3.3)	0.2196	0.1187	0.5732	0.1172
ABC irritability first evaluation	506	31.0 (11.9)	393	29.9 (11.4)	58	35.8 (14.1)	55	33.2 (11.8)	0.0006	0.0004	0.0473	0.2830
ABC social avoidance first evaluation	506	7.7 (3.4)	393	7.7 (3.4)	58	7.6 (3.4)	55	7.4 (3.4)	0.7930	0.8768	0.4987	0.6881
ABC social unresponsiveness first evaluation	506	18.8 (5.7)	393	18.5 (5.9)	58	19.9 (5.3)	55	19.6 (5.3)	0.1154	0.0811	0.1916	0.7393
ABC stereotypy first evaluation	506	10.9 (4.8)	393	10.5 (4.6)	58	12.7 (5.4)	55	11.9 (5.1)	0.0011	0.0010	0.0296	0.4655
ABC hyperactivity last evaluation	504	17.5 (6.9)	382	16.4 (6.2)	58	21.5 (7.2)	64	20.1 (8.3)	<0.0001	<0.0001	<0.0001	0.3237
ABC inappropriate Speech last evaluation	504	8.0 (3.4)	382	7.8 (3.4)	58	8.7 (3.5)	64	8.4 (3.4)	0.1310	0.0789	0.2204	0.6524
ABC irritability last evaluation	504	30.0 (11.7)	382	28.8 (11.1)	58	33.5 (12.3)	64	34.1 (13.0)	0.0002	0.0032	0.0007	0.8072
ABC social avoidance last evaluation	504	7.5 (3.4)	382	7.4 (3.3)	58	8.1 (3.8)	64	7.2 (3.3)	0.2760	0.1532	0.6003	0.1568
ABC social unresponsiveness last evaluation	504	18.4 (5.7)	382	18.2 (5.8)	58	19.0 (5.1)	64	19.5 (5.1)	0.1634	0.3221	0.0856	0.5669
ABC stereotypy last evaluation	504	10.5 (4.6)	382	10.1 (4.3)	58	11.8 (5.0)	64	12.0 (5.0)	0.0006	0.0061	0.0016	0.8347

**for the p-values listed for group comparisons, 1 = no seizures, 2 = seizures for < 3 years, 3 = seizures for > 3 years.*

of females with seizures in the cohort. Consequently, a larger cohort would be needed to confirm that females with FXS have a larger proportion of late-onset seizures than males. There is a new concern in adolescents and adults with idiopathic ASD of having new-onset seizures (43), especially in those with ID (44), which appears to be linked to increased morbidity (45, 46). Interestingly, we did not find this trend in those with FXS, making this less of a concern in this genetic disorder.

As with idiopathic ASD and many other ID syndromes, seizure type was variable between patients. However, the proportions of generalized and partial seizures are in general agreement with the literature (13–15, 19). Overall, the number of anticonvulsants required for those with seizures was low, consistent with past observations (15, 19). As might be expected considering that males with FXS are more affected than females, males with seizures were more likely to be on anticonvulsants, including multiple drugs, and to be followed in clinic and in FORWARD longer than females with seizures. The anticonvulsants used in the FXS population were consistent with recommendations in the field (2, 19, 47) and were those typically employed in any pediatric seizure population, and similar perhaps with somewhat less use of levetiracetam, most likely due to concerns about aggravating behavior.

For all participants with follow-up visits in FORWARD, new (i.e., incident) seizures were rare, with risk being only approximately 1% per patient year of follow-up in FORWARD. Risk of change in seizure type was only approximately 4% per patient year. The Kaplan–Meier plot (**Figure 4**) shows risk of having seizures at any given age across the lifespan. It is clear that most seizures are present early in life. After being stable through the 15–25 year age period with fewer patients having any seizure during this time period, the proportion of seizures goes up after age 25, most likely due to the small number of adults >25 years old included in FORWARD to date. There is also a likely referral bias such that patients are more likely to remain in care at an FXS clinic if they have ongoing seizures or be referred to an FXS clinic if they have new-onset seizures. This may be particularly the case for clinics run by pediatric neurologists, who tend to see adults with FXS more readily. Since the FORWARD project is now focused on increasing enrollment of adults, it is expected that more definitive estimates can be obtained for seizure prevalence and onset in adults upon future analysis of a larger cohort.

When patients with longer duration of seizures (>3 years) were compared with those with shorter duration (<3 years), only a few differences emerged, including increased use of anticonvulsants, higher prevalence of partial seizures, and lower prevalence of febrile seizures and, most importantly, more severe language outcomes despite lack of differences in behavioral abnormalities (including ASD) and non-significant increases in ID severity. This suggests that a longer course of seizures in FXS is an index of severity linked to more severe outcome in this some functional domains. It is possible that the division of the groups at 3 years of persistent seizures did not isolate those with the most severe phenotypes and a 5 year cutoff might have been better to more fully explore this concept. Nonetheless, there were not enough patients in FORWARD with 5 years of longitudinal data at present to support such an analysis.

Strengths of this study are the size of the subject sample, very large for rare disease standards, and the ability to examine longitudinal data. Weaknesses include lack of availability of follow-up visits into the late teenage years in about half the cohort, lack of complete evaluations of seizure type (many were undefined), and a relatively small population of adults in the current FORWARD database. Additionally, as participants with FXS were not a randomly selected, sampling biases inherent to clinic-based samples cannot be discounted.

Based on the data from this study, the prototypical individual with FXS and seizures would be male, with moderate or severe ID and expressive language delay, with a DSM-5 diagnosis of ASD and a corresponding SCQ >15 and SRS-2 in the severe range, who had a first evaluation at a FORWARD (perhaps any FXS) clinic after age 5. Females with FXS and seizures would show similar characteristics; however, among females a subgroup with a distinctive but uncommon profile of onset of seizures after age 15 is identified. It appears that this subgroup has greater cognitive impairment than other females with or without seizures and had a first evaluation at a FORWARD clinic after age 15. The duration of seizures also seems to be a parameter associated with worse outcomes, particularly in cognitive function.

FMRP has been linked to seizures in FXS through excessive mGluR5 signaling of dendritic translation, based on reversal of audiogenic seizures by mGluR5 blockers in the fmr1 knockout mouse (48). FMRP also operates presynaptically and may cause seizures through interruption of a direct interaction of FMRP with a presynaptic BK channel subunit (49). Seizures are associated with mutations in CYFIP2, which interacts with FMRP (50), and FMRP appears to underlie enhanced mLTD in adult rats triggered by early-life seizures (51). Thus, absence of FMRP may potentiate seizure *via* multiple neural mechanisms which are likely to vary between patients, thus resulting in variable penetrance and severity of seizures in FXS. However, likely the presence of seizures is a signal of more problematic neural and synaptic dysfunction related to variation in FMRP deficits in cells, and variation in these multiple interacting pathways. The present study adds to the understanding of the characteristics, risk factors, and course of seizures in FXS and provides a basis for anticipatory guidance for clinicians and families. The study also defines gaps and additional areas of investigation that could answer further questions about seizures, their neural underpinnings, and their evolution across the lifespan in FXS.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional IRBs at all FORWARD academic sites. Written informed consent to participate in this study was provided by the participant's legal guardian/next of kin.

FORWARD CONSORTIUM

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EB-K, RFi, RFr, SG, SM, HA, T-HC, and WK: study concept and design, acquisition, analysis, and interpretation of data, drafting and critical revision of the manuscript, and have approved it for publication. All authors contributed to the article and approved the submitted version.

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REFERENCES

1. Coffee B, Keith K, Albizua I, Malone T, Mowrey J, Sherman SL, et al. Incidence of fragile X syndrome by newborn screening for methylated FMR1 DNA. *Am J Hum Genet.* (2009) 85:503–14. doi: 10.1016/j.ajhg.2009.09.007
2. Kidd SA, Lachiewicz A, Barbouth D, Blitz RK, Delahunty C, McBrien D, et al. Fragile X syndrome: a review of associated medical problems. *Pediatrics.* (2014) 134:995–1005. doi: 10.1542/peds.2013-4301
3. Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP, Pizzuti A, et al. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell.* (1991) 65:905–14. doi: 10.1016/0092-8674(91)90397-H
4. Devys D, Lutz Y, Rouyer N, Bellocq JP, Mandel JL. The FMR-1 protein is cytoplasmic, most abundant in neurons and appears normal in carriers of a fragile X premutation. *Nat Genet.* (1993) 4:335–40. doi: 10.1038/ng0893-335
5. Volk LJ, Pfeiffer BE, Gibson JR, Huber KM. Mutiple Gq-coupled receptors converge on a common protein synthesis-dependent long-term depression that is affected in fragile X syndrome mental retardation. *J Neurosci.* (2007) 43:11624–34. doi: 10.1523/JNEUROSCI.2266-07.2007
6. Banerjee A, Ifrim MF, Valdez AN, Raj N, Bassell GJ. Aberrant RNA translation in fragile X syndrome: from FMRP mechanisms to emerging therapeutic strategies. *Brain Res.* (2018) 1693:24–36. doi: 10.1016/j.brainres.2018.04.008
7. Gross C, Hoffmann A, Bassell GJ, Berry-Kravis EM. Therapeutic strategies in fragile X syndrome: from bench to bedside and back. *Neurotherapeutics.* (2015) 12:584–608. doi: 10.1007/s13311-015-0355-9
8. He CX, Portera-Cailliau C. The trouble with spines in fragile X syndrome: density, maturity and plasticity. *Neuroscience.* (2013) 251:120–8. doi: 10.1016/j.neuroscience.2012.03.049
9. Chuang SC, Zhao W, Bauschwitz R, Yan Q, Bianchi R, Wong RK. Prolonged epileptiform discharges induced by altered group I metabotropic glutamate receptor-mediated synaptic responses in hippocampal slices of a fragile X mouse model. *J Neurosci.* (2005) 25:8048–55. doi: 10.1523/JNEUROSCI.1777-05.2005
10. Musumeci SA, Bosco P, Calabrese G, Bakker C, De Sarro GB, Elia M, et al. Audiogenic seizures susceptibility in transgenic mice with fragile X syndrome. *Epilepsia.* (2000) 41:19–23. doi: 10.1111/j.1528-1157.2000.tb01499.x
11. Kluger G, Bohm I, Laub MC, Waldenmaier C. Epilepsy and fragile X gene mutations. *Pediatr Neurol.* (1996) 15:358–60. doi: 10.1016/S0887-8994(96)00251-2
12. Musumeci SA, Ferri R, Elia M, Colognola RM, Bergonzi P, Tassinari CA. Epilepsy and fragile X syndrome: a follow-up study. *Am J Med Genet.* (1991) 38:511–13. doi: 10.1002/ajmg.1320380276
13. Musumeci SA, Hagerman RJ, Ferri R, Bosco P, Dalla Bernardina B, Tassinari CA, et al. Epilepsy and EEG findings in males with fragile X syndrome. *Epilepsia.* (1999) 40:1092–99. doi: 10.1111/j.1528-1157.1999.tb00824.x
14. Sabaratnam M, Vroegop PG, Gangadharan SK. Epilepsy and EEG findings in 18 males with fragile X syndrome. *Seizure.* (2001) 10:60–3. doi: 10.1053/seiz.2000.0492
15. Berry-Kravis E. Epilepsy in fragile X syndrome. *Dev Med Child Neurol.* (2002) 44:724–28. doi: 10.1111/j.1469-8749.2002.tb00277.x
16. Bailey DB, Raspa M, Olmsted M, Holiday DB. Co-occurring conditions associated with FMR1 gene variations: findings from a national parent survey. *Am J Med Genet.* (2008) 146A:2060–69. doi: 10.1002/ajmg.a.32439
17. Heard TT, Ramgopal S, Picker J, Lincoln SA, Rotenberg A, Kothare SV, et al. Abnormalities and seizures in genetically diagnosed fragile X syndrome. *Int J Dev Neurosci.* (2014) 38:155–60. doi: 10.1016/j.ijdevneu.2014.07.002
18. Haessler F, Gaese F, Huss M, Kretschmar C, Brinkman M, Peters H, et al. Characterization, treatment patterns, and patient-related outcomes of patients with Fragile X syndrome in Germany: final results of the observational EXPLAIN-FXS study. *BMC Psychiatry.* (2016) 16:318. doi: 10.1186/s12888-016-1020-5
19. Berry-Kravis E, Raspa M, Loggin-Hester L, Bishop E, Holiday D, Bailey D. Seizures in fragile X syndrome: characteristics and co-morbid diagnoses. *Am J Intellect Dev Disabil.* (2010) 115:461–72. doi: 10.1352/1944-7558-115.6.461
20. Rees M, Diebold U, Parker K, Doose H, Gardiner RM, Whitehouse WP. Benign childhood epilepsy with centrotemporal spikes and the focal sharp

wave trait is not linked to the fragile X region. *Neuropediatrics.* (1993) 24:211–13. doi: 10.1055/s-2008-1071542
21. Bonanni P, Casellato S, Fabbro F, Negrin S. Epilepsy in fragile-X-syndrome mimicking panayiotopoulos syndrome: description of three patients. *Am J Med Genet A.* (2017) 173:2753–57. doi: 10.1002/ajmg.a.38373
22. Gauthey M, Poloni CB, Ramelli GP, Roulet-Perez E, Korff CM. Status epilepticus in fragile X syndrome. *Epilepsia.* (2010) 51:2470–3. doi: 10.1111/j.1528-1167.2010.02761.x
23. McDermott S, Hardin JW, Royer JA, Mann JR, Tong X, Ozturk OD, et al. Emergency department and inpatient hospitalizations for young people with fragile X syndrome. *Am J Intellect Dev Disabil.* (2015) 120:230–43. doi: 10.1352/1944-7558-120.3.230
24. Kenmuir C, Richardson M, Ghearing G. Surgical treatment for medically refractory focal epilepsy in a patient with fragile X syndrome. *Brain Dev.* (2015) 37:916–8. doi: 10.1016/j.braindev.2015.02.009
25. Myers KA. van 't Hof FNG, Sadleir LG, Legault G, Simard-Tremblay E, Amor DJ, et al. Fragile females: case series of epilepsy in girls with FMR1 disruption. *Pediatrics.* (2019) 144:e20190599. doi: 10.1542/peds.2019-0599
26. Garcia-Nonell C, Ratera ER, Harris SW, Hessl D, Ono MY, Tartaglia N, et al. Secondary medical diagnosis in fragile X syndrome with and without autism spectrum disorder. *Am J Med Genet.* (2008) 146A:1911–16. doi: 10.1002/ajmg.a.32290
27. Cowley B, Kirjanen S, Partanen J, Castrén ML. Epileptic electroencephalography profile associates with attention problems in children with fragile X syndrome: review and case series. *Front Hum Neurosci.* (2016) 10:353. doi: 10.3389/fnhum.2016.00353
28. Sherman SL, Kidd SA, Berry-Kravis E, Andrews H, Lincoln S, Swanson M, et al. Fragile X online registry with accessible research database (FORWARD): experience from the fragile X clinical and research consortium to study the natural history fragile X syndrome. *Pediatrics.* (2017) 139:S183–93. doi: 10.1542/peds.2016-1159E
29. Aman MG, Singh NN, Stewart AW, Field CJ. The aberrant behavior checklist: a behavior rating scale for the assessment of treatment effects. *Am J Ment Defic.* (1985) 89:485–91. doi: 10.1037/t10453-000
30. Rutter M, Bailey A, Lord C. *The Social Communication Questionnaire: Manual*. Los Angeles, CA: Western Psychological Services (2003).
31. Constantino JN, Gruber CP. *The Social Responsiveness Scale-Second Edition*. Los Angeles, CA: Western Psychological Services (2012).
32. Sansone SM, Widaman KF, Hall SS, Reiss AL, Lightbody A, Kaufmann WE, et al. Psychometric study of the aberrant behavior checklist in fragile X syndrome and implications for targeted treatment. *J Autism Dev Disord.* (2012) 42:1377–92. doi: 10.1007/s10803-011-1370-2
33. Kaufmann WE, Kidd SA, Andrews HF, Budimirovic DB, Esler A, Haas-Givler B, et al. Autism spectrum disorder in fragile X syndrome: characterization using FORWARD. *Pediatrics.* (2017) 139:S194–206. doi: 10.1542/peds.2016-1159F
34. El Achkar CM, Spence SJ. Clinical characteristics of children and young adults with co-occurring autism spectrum disorder and epilepsy. *Epilepsy Behav.* (2015) 47:183–90. doi: 10.1016/j.yebeh.2014.12.022
35. Frye RE. Prevalence, significance and clinical characteristics of seizures, epilepsy and subclinical electrical activity in autism. *N A J Med Sci.* (2015) 8:113–22.
36. Abrahams BS, Geschwind DH. Advances in autism genetics: on the threshold of a new neurobiology. *Nat Rev Genet.* (2008) 9:341–55. doi: 10.1038/nrg2346
37. Lukmanji S, Manji SA, Kadhim S, Sauro KM, Wirrell EC, Kwon CS, et al. The co-occurrence of epilepsy and autism: a systematic review. *Epilepsy Behav.* (2019) 98:238–48. doi: 10.1016/j.yebeh.2019.07.037
38. Lewine JD, Andrews R, Chez M, Patil AA, Devinsky O, Smith M, et al. Magnetoencephalographic patterns of epileptiform activity in children with regressive autism spectrum disorders. *Pediatrics.* (1999) 104:405–18. doi: 10.1542/peds.104.3.405
39. Rossi PG, Parmeggiani A, Bach V, Santucci M, Visconti P. EEG features and epilepsy in patients with autism. *Brain Dev.* (1995) 17:169–74. doi: 10.1016/0387-7604(95)00019-8
40. Nass R, Gross A, Devinsky O. Autism and autistic epileptiform regression with occipital spikes. *Dev Med Child Neurol.* (1998) 40:453–8. doi: 10.1111/j.1469-8749.1998.tb15395.x
41. Capal JK, Carosella C, Corbin E, Horn PS, Caine R, Manning-Courtney P, et al. Endophenotypes in autism spectrum disorder. *Epilepsy Behav.* (2018) 88:341–8. doi: 10.1016/j.yebeh.2018.09.036
42. Frye RE, Butler I, Strickland D, Castillo E, Papanicolaou A. Electroencephalogram discharges in atypical cognitive development. *J Child Neurol.* (2010) 25:556–66. doi: 10.1177/0883073809344743
43. Tuchman R, Cuccaro M, Alessandri M. Autism and epilepsy: historical perspective. *Brain Dev.* (2010) 32:709–18. doi: 10.1016/j.braindev.2010.04.008
44. Bishop L, McLean KJ, Rubenstein E. Epilepsy in adulthood: prevalence, incidence, and associated antiepileptic drug use in autistic adults in a state Medicaid system. *Autism.* (2021) 25:831–9. doi: 10.1177/1362361320942982
45. Pickett J, Xiu E, Tuchman R, Dawson G, Lajonchere C. Mortality in individuals with autism, with and without epilepsy. *J Child Neurol.* (2011) 26:932–9. doi: 10.1177/0883073811402203
46. Hirvikoski T, Mittendorfer-Rutz E, Boman M, Larsson H, Lichtenstein P, Bölte S. Premature mortality in autism spectrum disorder. *Br J Psychiatry.* (2016) 208:232–8. doi: 10.1192/bjp.bp.114.160192
47. Hagerman RJ, Berry-Kravis E, Kaufmann WE, Ono MY, Tartaglia N, Lachiewicz A, et al. Advances in the treatment of fragile X syndrome. *J Pediatr.* (2009) 123:378–90. doi: 10.1542/peds.2008-0317
48. Yan QJ, Rammal M, Tranfaglia M, Bauchwitz RP. Suppression of two major fragile X Syndrome mouse model phenotypes by the mGluR5 antagonist MPEP. *Neuropharmacology.* (2005) 49:1053–66. doi: 10.1016/j.neuropharm.2005.06.004
49. Myrick LK, Deng PY, Hashimoto H, Oh YM, Cho Y, Poidevin MJ, et al. Independent role for presynaptic FMRP revealed by an FMR1 missense mutation associated with intellectual disability and seizures. *Proc Natl Acad Sci U S A.* (2015) 112:949–56. doi: 10.1073/pnas.1423094112
50. Zweier M, Begemann A, McWalter K, Cho MT, Abela L, Banka S, et al. Spatially clustering de novo variants in CYFIP2, encoding the cytoplasmic FMRP interacting protein 2, cause intellectual disability and seizures. *Eur J Hum Genet.* (2019) 27:747–59. doi: 10.1038/s41431-018-0331-z
51. Bernard PB, Castano AM, O'Leary H, Simpson K, Browning MD, Benke TA. Phosphorylation of FMRP and alterations of FMRP complex underlie enhanced mLTD in adult rats triggered by early life seizures. *Neurobiol Dis.* (2013) 59:1–17. doi: 10.1016/j.nbd.2013.06.013

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Cardiac Startle Response and Clinical Outcomes in Preschool Children With Fragile X Syndrome and Autism Spectrum Disorder

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Objective: Poor physiological regulation in response to threat is linked to multiple negative developmental outcomes including anxiety, which is highly prevalent and impairing in young children with neurodevelopmental disabilities like fragile X syndrome (FXS) and autism spectrum disorder (ASD). The present study contrasted cardiac startle response in pre-school-aged children with FXS, with and without ASD, to children with non-syndromic ASD (nsASD) and neurotypical controls (NT). The relationship of cardiac startle to non-verbal mental age (NVMA), ASD severity, and parent-reported anxiety was also examined.

Method: Four age-matched groups of pre-school children participated including those with FXS without ASD (FXS-Only, $n = 21$), FXS with ASD (FXS+ASD, $n = 17$), nsASD ($n = 42$), and NT children ($n = 27$). Participants viewed a silent movie during which a single 200 ms 98-decibel white noise burst occurred. Cardiac activity was analyzed for pre-stimulus respiratory sinus arrhythmia (RSA) and the inter-beat intervals (IBI) at the auditory stimulus and 10 s post-stimulus. The Spence Pre-school Anxiety Scale, Autism Diagnostic Observation Schedule-2nd Edition, and Mullen Scales of Early Learning were examined in relation to startle response.

Results: The nsASD group demonstrated heightened cardiac activity at the auditory stimulus and 10 s post-stimulus compared to the NT controls. Neither of the FXS groups showed differences from any other group. Higher pre-stimulus RSA was associated with reduced cardiac response across groups, while the relationship between cognitive ability and ASD severity to cardiac response varied between groups. Parent-reported anxiety was not associated with cardiac response for any group.

Conclusion: These findings demonstrate group distinctions in cardiac responses to auditory startle. Although FXS and ASD share behavioral characteristics, the nsASD group showed a heightened cardiac startle response compared to the NT group that was not present in the FXS groups with or without ASD. Non-verbal mental age was associated with greater stimulus or post-stimulus reactivity for all groups except the

FXS+ASD group, which showed no association between startle response and any clinical outcomes. Increased understanding of the relationship between physiological regulation and clinical outcomes will assist in identifying the timing and targets for effective interventions for individuals with neurodevelopmental disabilities.

Keywords: pre-school, intellectual disabilities (ID), anxiety, heart activity, physiological startle, autism spectral disorder (ASD), fragile X syndrome

INTRODUCTION

Physiological regulation during threat is a critical adaptive response formed early in development. When a threat is experienced, the autonomic nervous system (ANS) activates the sympathetic branch in response, while the parasympathetic branch supports the body in recovering to a baseline state after the threat has passed. Well-integrated physiological regulation is related to a range of positive outcomes including better language skills, increased social responsiveness, peer engagement, emotion recognition, healthy social attachment, and social approach (1, 2). In contrast, physiological dysregulation is linked to a litany of maladaptive outcomes including emotion dysregulation, social deficits, delayed adaptive skills, and a range of psychological disorders (3, 4). Specifically, a heightened physiological response and reduced modulation in response to threat, like an auditory startle, can be indicative of anxiety (5, 6). Individuals with neurodevelopmental disabilities are at an elevated risk for physiological dysregulation and emotional difficulties, despite differing etiologies. Because there is a clear relationship between physiological regulation and developmental outcomes (7, 8), studying this phenomenon in individuals with neurodevelopmental disabilities can provide unique insights into the biological mechanisms of anxiety.

One way to capture physiological regulation is to measure heart activity in response to threat. Two cardiac indices of physiological regulation are inter-beat interval (IBI), defined as the time between heartbeats and an indicator of heart rate, and respiratory sinus arrhythmia (RSA), the temporal variation in IBI synced with respiration. When faced with an unpredictable or sudden threat, a specific pattern of physiological reactivity, known as the startle response, is often observed. This startle response is considered a primitive, elicited response to intense or sudden stimuli that prepares the body for "fight or flight" (9). During a startle response, the sympathetic nervous system is activated, allowing blood flow to move more rapidly to the extremities and breathing to increase, which allows the body to mobilize a response. During this phase, both reduced IBI (i.e., less time between heartbeats), and reduced RSA (i.e., less variability in the time between heartbeats) are typically observed. After the threat has passed, the parasympathetic nervous system becomes activated, which causes breathing to slow and blood flow to return to central organs (10). Additionally, IBI and RSA both increase as they return to baseline "resting state" levels.

Individuals with anxiety often show an atypical physiological startle response. For example, adults and children with anxiety exhibit lower resting RSA, which leads to the sympathetic system over-responding to threat, causing an exaggerated startle reflex of increased muscle tension, blink response, RSA withdrawal, and galvanic skin response. Individuals with lower resting RSA also show a slower return to baseline state after a threat (5, 6, 11). Additionally, children with anxiety often exhibit a shorter IBI at resting state and a slower IBI recovery than non-anxious peers, suggesting that they have restricted autonomic flexibility in response to threatening stimuli (4, 8, 12). Evidence also suggests that lower RSA during a resting state is predictive of a heightened startle response in typical adults (11). Taken together, these studies provide compelling evidence that physiological dysregulation, particularly in response to threat, may underlie vulnerability to anxiety in neurotypical individuals. However, little work has examined IBI and RSA during a startle paradigm in children with neurodevelopmental disorders, such as autism spectrum disorder (ASD) or fragile X syndrome (FXS).

Children with neurodevelopmental disorders appear to be at elevated risk for both physiological dysregulation and anxiety. Autism spectrum disorder (ASD) is a neurodevelopmental disorder characterized by deficits in social communication skills and the presence of restricted, repetitive behaviors (13). Current estimates suggest that 1 in 54 children have an ASD, and ∼40–50% will develop a co-occurring anxiety disorder (14–16). Fragile X Syndrome (FXS) is a monogenic disorder characterized by atypical social and communication skills, repetitive behaviors, and intellectual disability. Approximately 1 in 4,000 males and 1 in 8,000 females have the full mutation of FXS, which results from a mutation on the *FMR1* gene of >200 CGG repeats (17, 18). Similar to children with ASD, children with FXS are also at a heightened risk for developing comorbid anxiety disorders (50–86%) (19, 20). Children with FXS also exhibit a behavioral phenotype that is strikingly similar to ASD, with ∼60% of children with FXS also meeting diagnostic criteria for ASD (21, 22). For instance, repetitive speech and behaviors, social avoidance, aberrant eye contact, and physiological dysregulation are features common to both ASD and FXS. Given the similar behavioral phenotypes but divergent etiology between non-syndromic ASD (nsASD) and FXS, cross-population studies can provide insight into genetic contributions to physiological and emotional dysregulation (2).

Studies of children with ASD suggest that autonomic dysregulation is common in this population with findings showing a persistent state of hyperarousal, though the pattern of findings are not consistent. For example, at baseline, most studies have found that individuals with ASD are hyper-aroused, showing lower RSA and higher heart rate than peers, yet some

studies found no differences (2, 23). Physiological responses to threat also appear to be atypical but varied in ASD, as evidenced by hyperarousal during cognitive tasks (elevated heart rate, lower RSA), no differences in heart rate during social interactions, and a blunted heart rate during social performance tasks (lower during stress) (24–26).

Some evidence suggests that features comorbid with ASD can also influence physiological response to threat, including anxiety and intellectual disability (ID). The role of anxiety in physiological responses to stress within ASD is limited and not well-understood. In studies of children and adolescents with ASD and comorbid anxiety, a blunted physiological response to threat (i.e., lower heart rate, less electrodermal activity) has been found compared to individuals with ASD only and typically developing peers (25, 27). In addition to anxiety, differences are also evident in individuals with ASD and ID in that some studies indicate that these individuals are hyper-aroused and show little variation in HR in response to stimuli (23). Combined, these findings suggest that the coordination of the sympathetic and parasympathetic nervous system's threat response is disrupted in some way, particularly in those children who also have anxiety or ID alongside ASD. Understanding the characteristics and outcomes of children with poor physiological regulation is important because reduced resting RSA has been associated with poorer language skills and social responsiveness in children with ASD. Overall, evidence suggests that physiological regulation has critical implications for downstream social-communicative functioning (3, 23, 28).

Evidence for physiological dysregulation is generally more consistent in children with FXS than in children with ASD. Overall, individuals with FXS show a developmental effect that becomes more pronounced with age, from hypo-arousal in the first 2 years of life toward hyperarousal thereafter, exhibiting reduced IBI and RSA during resting state (2, 29). In response to threat, one study found that infants with FXS showed reduced RSA during a stranger approach paradigm, a task designed to elicit social fear where an examiner dresses in a disguise and approaches the child (30). Another study found no differences in change in IBI in response to an auditory startle between boys with FXS and neurotypical boys, but did find that older boys with FXS showed a stronger startle response than younger boys with FXS (31). Similarly, in another study examining heart activity during rest and stress, adolescents with FXS remained in an aroused state throughout rest and stress periods (i.e., reduced RSA, shorter IBI) compared to typically developing peers (32). Thus, it appears that children with FXS exhibit chronic hyperarousal through baseline arousal as well as response to cognitive or social threats, which becomes more pronounced over age. However, the link between the physiological startle response and anxiety has not been investigated in FXS. Further, information regarding the potential impacts of co-occurring ASD on physiological dysregulation in FXS is limited, although evidence suggests that individuals with ASD+FXS often have behavioral differences and decreased cognitive and adaptive functioning (21, 33). Given the differences in developmental profiles, a direct comparison of startle response between children with FXS with and without ASD could provide insight into underlying physiological differences.

Although a link between physiological response to threat and anxiety has been established in neurotypical populations, it remains understudied in clinical groups at elevated risk for anxiety, such as ASD and FXS. A clearer understanding of the cardiac startle response in individuals with neurodevelopmental disabilities and how it relates to clinical features can provide insight into the mechanistic underpinnings of negative behavioral outcomes and guide the development of targeted prevention and intervention programs. The present study is the first to assess the cardiac startle response in pre-school-aged children with nsASD, FXS with comorbid ASD (FXS+ASD), and FXS only (FXS-Only) compared to neurotypical peers (NT). Our specific research questions are as follows:

1a. Are there differences in cardiac response (IBI) to threat during an auditory startle paradigm between pre-school children with nsASD, FXS+ASD, and FXS-Only compared to NT controls? 1b. Does pre-startle RSA predict startle IBI differentially across groups?
2. Does cardiac response to startle (IBI) relate to non-verbal mental age, ASD severity, or parent-reported anxiety, and do these relationships differ by group?

Given the current evidence for cardiac dysregulation in both FXS and ASD, it is anticipated that the clinical groups will demonstrate an exaggerated physiological response to threat (shorter IBI) after an unexpected auditory stimulus compared to the neurotypical (NT) controls. Additionally, it is expected that higher baseline RSA will be associated with longer IBI during the auditory stimulus, regardless of group. Exaggerated startle is expected to be related to clinical outcomes including low non-verbal mental age, high ASD severity, and high anxiety symptoms, given the connection between poor regulation and negative outcomes.

METHODS

Participants

Participants for this study were drawn from an ongoing NIMH study (1R01MH107573-01A1; PI: Roberts) that is focused on the emergence of anxiety symptoms in young children with neurodevelopmental disabilities. The present sample consisted of 107 children between 36 and 72 months of age divided into four diagnostic groups: non-syndromic ASD (nsASD; $n = 42$, 36 males), FXS with comorbid ASD ruled out (FXS-Only; $n = 21$, 11 males), FXS with comorbid ASD (FXS+ASD; $n = 17$, 13 males), and neurotypical controls (NT; $n = 27$, 20 males). Participants were excluded if they were born premature (gestational age $<$ 37 weeks) or had a history of seizures. Participants with FXS were confirmed to have the full mutation ($>$200 CGG repeats) of the *FMR1* gene through genetic records. The nsASD group had no known genetic disorders. Autism diagnoses for the nsASD and FXS+ASD groups were confirmed through a Clinical Best Estimate (CBE) review process (22). The NT sample had no known diagnoses nor family history of ASD. The FXS-Only and NT samples were confirmed to not have ASD through the CBE process. Both males and females were included in the study in order to reflect the heterogeneity of the populations.

Measures

Auditory Startle Paradigm

The Anxiety Dimensional Observation Schedule (Anx-DOS) (34) is an observational measure that consists of a variety of tasks designed to elicit anxious and fearful behaviors in pre-school-aged children. During the auditory startle task, each participant watched a two-and-a-half-minute silent children's movie while wearing a heart rate monitor. Approximately halfway through the movie, a 200 ms white noise burst occurs at ∼98 decibels.

Physiological Regulation

Heart activity data was recorded continuously through two electrodes placed onto the child's chest using the Actiwave Cardio Monitor (CamNtech Ltd., Cambridge, UK) at 1,024 Hz. To ensure uniformity among participants, a trained research assistant identified the heart activity data period of interest as 30 s prior to the auditory stimulus through 90 s after. The cropped heart activity data was visually inspected and edited off-line for artifacts, arrhythmias, and false heart periods by trained research assistants using CardioEdit software (Brain-Body Center, University of Illinois at Chicago). Mean values for RSA and IBI were extracted using CardioBatch software (Brain-Body Center, University of Illinois at Chicago). To calculate RSA values, CardioBatch samples sequential heart periods at 250 ms epochs and then de-trends the data with a 21-point moving polynomial algorithm (35). The data was then bandpassed filtered to extract variance associated with spontaneous breathing parameters (0.24–1.04 Hz). The variance was then changed to its natural logarithm to provide an estimate of RSA.

Pre-Stimulus RSA was defined as the mean RSA for the 30 s prior to the auditory stimulus as a measure of baseline RSA. Stimulus IBI was the mean IBI extracted from the 1-s interval that began at the onset of the auditory stimulus. Post-stimulus IBI was the mean IBI extracted from the 1-s interval at 10 s post-stimulus (**Figure 1**). Pre-stimulus RSA assessed the capacity for regulation during the startle, while stimulus and post-stimulus IBI captured the acute cardiac startle response.

Autism Diagnosis and Severity

The *Autism Diagnostic Observation Schedule- 2nd Edition* (ADOS-2) is a semi-structured, play-based observational measure assessing the presence of autism symptomology (36). The tasks in the ADOS-2 are designed to elicit social-communication skills, and the presence of restricted, repetitive behaviors. The ADOS-2 has four modules (1–4), ranging from non-verbal to fluent verbal abilities. The ADOS-2 was administered and scored by research reliable, graduate-level professionals and reviewed by a licensed psychologist through the CBE process to confirm an ASD diagnosis in the ASD group. Site reliability was conducted on 20% of the ADOS-2 administrations (item-level inter-rater agreement = 83.3%). The Calibrated Comparison Score (CSS) is an overall severity composite relative to children of similar language abilities, ranging from 1 to 10. The CSS was used as a continuous variable of ASD severity across groups for analysis in the present study.

Anxiety Symptoms

The *Spence Pre-school Anxiety Scale* (PAS) (37) is a 34-item caregiver report of anxiety symptoms in children aged 2.5–6.5 years. Item scores range from 0 to 4 and summary scores are computed for Generalized Anxiety, Social Anxiety, Obsessive-Compulsive disorder, Physical Injury Fears, Separation Anxiety, and Total Anxiety. For this study, the Total Anxiety raw score was used as a measure of overall anxiety symptoms. While the PAS was developed for typically developing children, studies suggest that it is an appropriate tool for parent-reported anxiety for children with ASD because many questions target observable behaviors (38–40).

Developmental Level

The *Mullen Scales of Early Learning* (MSEL) (41) is a standardized measure designed to assess development from birth to 68 months across gross and fine motor skills, receptive and expressive language, and visual reception. Children with ASD and FXS often have language delays that can overshadow cognitive skills, particularly before age 5 (42). Evidence suggests that non-verbal IQ is a more stable and accurate representation of cognitive ability in young children with ASD (43). Thus, non-verbal mental age (NVMA) was used as an index of non-verbal cognitive ability for the present study. NVMA was computed by averaging the visual reception age equivalent and the fine motor age equivalent ($\frac{VR\ age + FM\ age}{2}$) (44). The MSEL shows good internal consistency for each subscale (0.75–0.08), and test-retest reliability (0.70–0.80).

Analytic Plan

Statistical analyses were conducted through three phases: preliminary analysis, stimulus analysis (RQ1), and correlates of startle response (RQ2). First, in the preliminary analysis, groups were compared through one-way analyses of variance (ANOVAs) to assess for group differences in chronological age, NVMA, ADOS-2 CSS, and PAS Total Anxiety scores. Pre-stimulus IBI was also compared through a one-way ANOVA to assess for group differences in IBI prior to the auditory stimulus (i.e., baseline IBI). Second, to evaluate the cardiac response to the auditory stimulus across a 90-s task (RQ1a), multilevel regression models were run to assess group differences in IBI during the 1-s period that began at the onset of the auditory stimulus and the 1-s period occurring 10 s post-stimulus to test time-by-group interactions. The interaction was probed by centering time at the stimulus and at 10 s post-stimulus to determine any points of significant divergence in IBI trajectories (45). Because IBI increases during the pre-school developmental period, groups were matched on chronological age. Then, a multiple regression model was run to assess if pre-startle RSA predicted startle IBI with group as a covariate (RQ1b). Finally, a within-group bivariate correlations were used to determine patterns of association between PAS Total Anxiety scores, ADOS-2 CSS, or NVMA and IBI at the stimulus and post-stimulus across each group (RQ2).

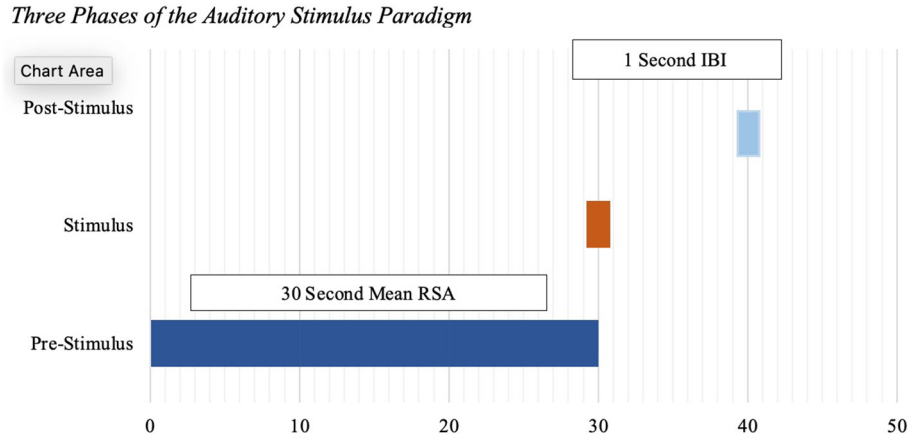

FIGURE 1 | Three phases of the auditory stimulus paradigm.

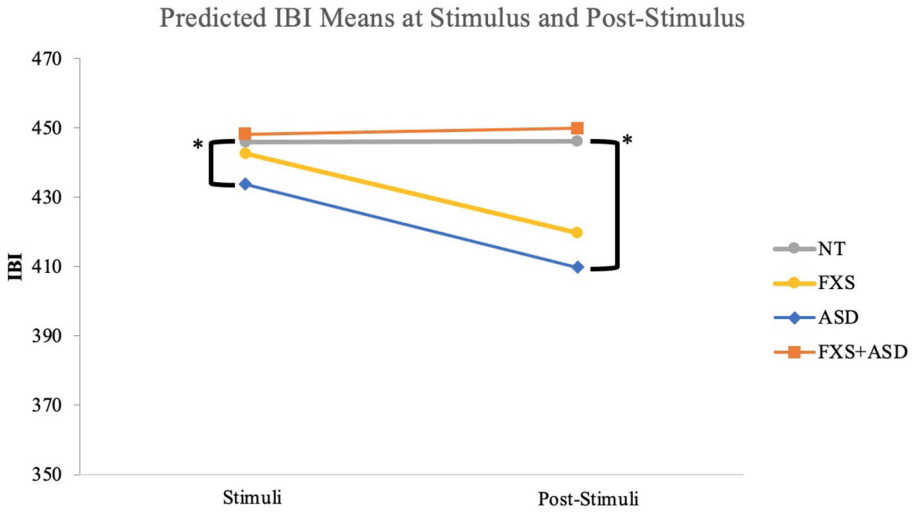

FIGURE 2 | Epoch by group comparison of inter-beat intervals during startle paradigm. *Significant differences were seen between the ASD and the NT groups for stimulus ($p = 0.03$) and post-stimulus IBI ($p = 0.02$).

RESULTS

Preliminary Analysis: Group Comparisons for Age, ADOS-2 CSS, NVMA, Spence Anxiety, and Pre-stimulus IBI

Results of the first one-way ANOVA showed no significant group differences for chronological age [$F_{(3,106)} = 0.14$; $p = 0.94$]. As anticipated, significant differences were found for ADOS-2 CSS between groups [$F_{(3,92)} = 78.8$; $p < 0.001$], in that the nsASD and FXS+ASD groups showed significantly higher severity scores than the FXS-Only and NT groups, and the FXS-Only group showed higher severity scores than the NT group. Results of the one-way ANOVA for NVMA also showed significant differences between groups [$F_{(3,102)} = 24.3$; $p < 0.001$]. As expected, the NT group demonstrated significantly higher NVMA than the FXS + ASD, ASD, and FXS-Only groups, and the FXS+ASD group showed significantly lower NVMA than all groups. The nsASD and FXS-Only groups were not significantly different on NVMA. For the PAS Total Anxiety Raw score, no significant group differences were observed [$F_{(3,92)} = 1.48$, $p = 0.23$]. Lastly, results of the one-way ANOVA indicated that groups did not differ on pre-stimulus IBI [$F_{(3,102)} = 1.25$, $p = 0.29$]. **Table 1** depicts means and standard deviations for each group.

Startle Analysis (RQ 1a): Group Comparisons for IBI at Stimulus and Post-stimulus

Multilevel regression model results with time centered at the stimulus (30 s) indicated that the nsASD group exhibited a significantly shorter IBI than the NT group ($b = -33.856$,

TABLE 1 | Descriptive data by group.

	Gender M:F	Age in months Mean (*SD*)	ADOS-2 CSS Mean (*SD*)	NVMA Mean (*SD*)	Spence raw total Mean (*SD*)	Pre-stimulus IBI Mean (*SD*)	Pre-stimulus RSA Mean (*SD*)
nsASD ($n = 42$)	36:6	46.08 (8.0)	7.09 $(1.4)_a$ ($n = 35$)	30.24 $(11.2)_b$ ($n = 40$)	12.17 (10.0) ($n = 35$)	574.06 (73.48)	5.90 (1.44)
FXS-only ($n = 21$)	11:10	47.46 (7.7)	3.56 $(1.8)_b$ ($n = 18$)	35.08 $(10.3)_b$ ($n = 20$)	14.44 (11.1) ($n = 18$)	577.18 (57.56)	6.08 (1.25)
FXS + ASD ($n = 17$)	13:4	46.10 (9.3)	7.33 $(1.5)_a$ ($n = 15$)	22.50 $(5.9)_c$ ($n = 16$)	15.47 (9.3) ($n = 15$)	583.71 (105.29)	5.20 (1.99)
NT ($n = 27$)	20:7	46.55 (9.2)	2.0 $(1.2)_c$ ($n = 25$)	47.59 $(10.7)_a$ ($n = 27$)	9.8 (6.2) ($n = 25$)	584.89 (76.40)	6.30 (1.39)

Group differences ($p < 0.05$) are indicated by different subscripts.

TABLE 2A | Regression model centered at stimulus.

	b	SE(*b*)	*P*
Intercept	609.445	13.190	<0.001
Epoch 30 s	0.014	0.014	0.90
FXS + ASD	−25.850	21.22	0.23
FXS-only	−27.508	19.94	0.17
nsASD	−35.501	16.91	0.038
FXS + ASD x Epoch 30 s	−0.003	0.17	0.99
FXS-only x Epoch 30 s	0.060	0.16	0.71
nsASD x Epoch 30 s	−0.126	0.14	0.36

TABLE 2B | Regression model centered at post-stimulus regression.

	b	SE(*b*)	*P*
Intercept	609.59	13.25	<0.001
Epoch 40 s	0.014	0.11	0.90
FXS + ASD	−25.88	21.31	0.23
FXS-only	−26.91	20.03	0.18
nsASD	−36.76	16.98	0.032
FXS + ASD x Epoch 40 s	−0.003	0.17	0.99
FXS-only x Epoch 40 s	0.060	0.16	0.71
nsASD x Epoch 40 s	−0.126	0.14	0.36

$p = 0.027$). Neither the FXS-Only group nor the FXS+ASD groups showed significant trajectories from the NT group with time centered at the stimulus (see **Table 2A**; represented in **Figure 2**). Results from probing group-by-time interactions at 10 s post-stimulus (40 s) indicated that the nsASD group continued to display a shorter IBI than the NT group ($b = -35.120$, $p = 0.026$). Further, the FXS-Only group and the FXS+ASD groups continued to show similar IBIs to the NT at 10 s post-stimulus. The interaction of IBI-by-time was not significant indicating that this group difference was present regardless of time (see **Table 2B**). The reference group was recoded in subsequent models to yield estimates for differences between the clinical groups in cardiac reactivity. These results indicated no significant differences between the three clinical groups for IBI at the stimulus or post-stimulus ($ps > 0.594$).

Relationship Between Pre-stimulus RSA and Stimulus IBI (RQ 1b)

Results from the multiple regression model assessing differential associations of pre-stimulus RSA to stimulus IBI as a function of group accounted for ∼49% of the variance in stimulus IBI. Additionally, parameter estimates indicated that accounting for group, pre-stimulus RSA was a predictor for stimulus IBI ($b = 41.68$; $p < 0.001$) such that for each unit increase in pre-stimulus RSA, there was an associated increase of 41.7 ms in IBI. Further, a lack of significant group-by-RSA interaction terms indicated that the effect of pre-stimulus RSA on stimulus IBI did not differ as a function of group (see **Table 3**).

TABLE 3 | Regression model of pre-stimulus RSA to stimulus IBI.

	b	SE(*b*)	*p*
Intercept	352.40	56.52	<0.001
Pre-stimulus RSA	41.68	8.80	<0.001
FXS + ASD	−69.71	70.88	0.33
FXS-only	42.34	88.65	0.63
nsASD	28.69	69.54	0.68
FXS + ASD x Pre-stimulus RSA	18.74	11.70	0.11
FXS-only x Pre-stimulus RSA	−11.03	14.10	0.44
nsASD x Pre-stimulus RSA	−8.13	11.05	0.46

Association Between Startle Response and Non-verbal Mental Age, ASD Severity, and Parent-Reported Anxiety (RQ 2)

Lastly, Pearson correlations were tested to assess if non-verbal mental age, ASD severity, or parent-reported anxiety predicted stimulus IBI or post-stimulus IBI within each group. NVMA was significantly correlated with stimulus IBI for the NT group ($r = 0.63$, $p < 0.001$) and FXS-Only group ($r = 0.50$, $p = 0.022$) and for post-stimulus IBI for the NT group ($r = 0.53$, $p = 0.004$) and for the ASD group ($r = 0.36$, $p = 0.022$). The FXS+ASD

TABLE 4 | *Post-hoc* correlations.

		ADOS-2 CSS	NVMA	Spence PAS
nsASD	Startle IBI	$r = -0.13$	$r = 0.26$	$r = 0.22$
	Post-startle IBI	$r = -0.10$	$r = 0.36^{*}$	$r = 0.23$
FXS-only	Startle IBI	$r = 0.44$	$r = 0.50^{*}$	$r = -0.17$
	Post-startle IBI	$r = 0.53^{*}$	$r = 0.42$	$r = -0.08$
FXS + ASD	Startle IBI	$r = -0.19$	$r = 0.12$	$r = -0.07$
	Post-startle IBI	$r = -0.28$	$r = 0.29$	$r = -0.02$
NT	Startle IBI	$r = 0.27$	$r = 0.63^{**}$	$r = -0.13$
	Post-startle IBI	$r = 0.22$	$r = 0.53^{**}$	$r = -0.23$

*Significant at $p < 0.05$; ** Significant at $p < 0.01$.

group did not show an association between NVMA and cardiac response to stimulus ($p > 0.273$). ADOS-2 CSS was moderately correlated with stimulus IBI ($r = 0.44$, $p = 0.07$) and significantly correlated with post-stimulus IBI ($r = 0.53$, $p = 0.02$) for the FXS-Only group, but no relationship was found for any other groups. Lastly, PAS Total Anxiety Raw Score was not significantly correlated with stimulus IBI or post-stimulus IBI for any group (**Table 4**).

DISCUSSION

Poor physiological regulation in response to threat is linked to a range of negative developmental outcomes including anxiety, behavioral difficulties, and low adaptive skills, which are highly prevalent and impairing in young children with neurodevelopmental disabilities, like FXS and ASD (2–4). In this study, we examined the cardiac response to an auditory startle in an age-matched sample of pre-school children with FXS, ASD, and typical development. We also investigate clinical features that are thought to be associated with poor physiological regulation. Our results indicate that the children with ASD exhibit an exaggerated cardiac response to a sudden auditory stimulus that differentiated them from NT children, but no other significant group differences were observed. Elevated pre-startle RSA was associated with longer IBI at the stimulus across all the groups. In contrast, more severe ASD symptoms were associated with reduced cardiac startle only in the group with FXS that did not have ASD. Also, elevated non-verbal ability was related to reduced cardiac startle in the NT, nsASD, and FXS group without ASD but not in the FXS group with ASD. Finally, parent-reported anxiety symptoms were not associated with cardiac startle in any group.

Cardiac Auditory Startle Responses Across Groups

The present study found that pre-school children with nsASD showed greater cardiac startle to an auditory startle relative to NT peers. Our findings are consistent with previous studies of individuals with ASD that indicate physiological dysregulation within this population (2, 23, 46, 47). While abnormalities in arousal are consistent in ASD, specific patterns of physiological response to stress are varied, possibly reflecting the heterogeneity of the ASD population. The present study is the first to assess cardiac startle response in young children with ASD and ID, as the majority of previous studies were conducted with individuals with higher cognitive and language abilities or older individuals with ASD. Given the heterogeneity of ASD populations, demonstrating hyperarousal in a relatively homogeneous sample of pre-school children with ASD and ID compared to an age-matched sample shows that this trend toward hyperarousal begins early in life.

Understanding patterns of physiological activity in children with ASD is important, as hyperarousal has been theorized to underlie behavioral and learning difficulties often present in ASD. A review by White et al. (47) posits that physiological hyperarousal in ASD is linked to emotion dysregulation, which can lead to social and psychological difficulties like anxiety. Emotional and behavioral problems can impact early learning and compound existing developmental delays and deficits. For instance, children with FXS and ASD often have more delays early in infancy and behavioral difficulties that cause long-term impairment, including the ability to become independent (48–53). It is essential to understand the presentation of physiological abnormalities in ASD early in life in order to improve developmental outcomes, including social and emotional health (23, 54).

Our findings show interesting parallels with two studies assessing startle responses in individuals with FXS. First, a study by Cohen et al. (45) compared physiological reactivity (electrodermal, heart activity, eye blink) in males aged 10–17 years old with ASD, FXS+ASD, FXS-Only, and NT controls as they viewed emotional stimuli. Similar to the present study, Cohen et al. (45) found that the two FXS groups showed similar patterns of cardiac reactivity, despite the presence of ASD. In contrast, in the Cohen et al. study (45), the FXS groups showed higher cardiac activity than the ASD group, whereas our results identified elevated cardiac activity in the nsASD group compared to the FXS groups. Because the present study consisted of young children, one possible explanation for differences is increased arousal over age in individuals with FXS (2, 29). Further, the present study included females and children with intellectual disabilities, which warrants continued investigation into these factors and the influence they have on cardiac reactivity in individuals with ASD and FXS.

The second study assessed the cardiac response to an auditory startle in boys with FXS aged 1–10.5 years old compared to a NT age-matched group (31). Similar to the present study, the Roberts et al. (31) results did not find significant differences in cardiac response to startle between the boys with FXS and the NT boys. The study did find that as children with FXS aged, their cardiac arousal to the startle increased, a shift not seen in the NT group. Although the developmental shift toward hyper-arousal with age for individuals with FXS was not seen in the present study, the sample was limited to pre-school-aged children and was cross-sectional, rather than longitudinal. Further, the present study examined the cardiac response in a sample of children with FXS divided into those with and without comorbid ASD, which may have implications for the trajectory of arousal in

FXS. Future studies should examine the longitudinal patterns of cardiac response to threat in these two groups for nuances in trajectories as children age into adolescence and the potential impacts of comorbid ASD.

Relationship Between Cardiac Auditory Startle and Clinical Features

In the second aim, we examined whether clinical features of ASD and FXS are associated with cardiac startle responses within and across groups. Cognitive level is particularly important to consider, as autonomic regulation has been linked to aspects of development, like language ability and adaptive functioning, but has not been assessed directly with cardiac startle in young children with intellectual disabilities. Interestingly, NVMA was strongly correlated with post-startle IBI in that, as non-verbal abilities increased, heart rate decreased. The NT group showed a moderate positive relationship between non-verbal ability and startle and post-startle IBI, suggesting that non-verbal abilities and the physiological regulation during an auditory startle are moderately linked in typical pre-schoolers. One hypothesis is that children with higher non-verbal cognitive abilities are able to regulate their cardiac response better than children with lower cognitive abilities because they can interpret the startle as non-threatening. Children with lower cognitive abilities, however, showed difficulty modulating their cardiac responses to the startle, suggesting that cognitive delays negatively impact physiological regulation to threat.

Within the clinical groups, the FXS-Only group also showed positive correlations between non-verbal ability and startle response like the NT group, while the nsASD group and the FXS+ASD group showed no relationship between NVMA and startle response. The shared behavioral diagnosis of ASD might indicate that features of ASD are confounding or influencing the relationship between cognitive ability and startle response. Some studies have found that cardiac flexibility is positively related to cognitive ability in ASD, whereas individuals with ASD and low cognitive abilities show higher physiological arousal and less flexibility in response to threat (23). Overall, the relationship between physiological response to startle and cognitive ability suggests that developmental delays are connected to cardiac startle in that higher non-verbal ability might support the ability to regulate physiologically because of the ability to cognitively cope in response to the startle.

Autism severity also significantly impacts development and the ability to regulate in response to stressors. The link between ASD severity and poor RSA is well-established (1, 55) and the clinical overlap of anxiety symptoms and features of ASD has been observed in neurotypical and ASD populations (55–58). Even with these established connections, no studies have assessed the relationship between cardiac response to startle and ASD severity within ASD and FXS samples. In the present study, the FXS-Only group showed a significant relationship between post-startle heartrate and ASD severity in that the higher the severity score, the less reactive the heartrate. This relationship was specific to the FXS-Only group, which uniquely represents a diagnostic group with ASD symptoms but not ASD. In the FXS-Only group, ASD severity might represent aspects of ASD behaviors like social avoidance or repetitive and restrictive behaviors that suggest a link between specific ASD features and decreased cardiac startle. Interestingly, neither the ASD+FXS group nor the nsASD group showed a relationship between ASD severity and startle response, suggesting that having ASD might overpower any relationship between ASD severity and startle response.

Lastly, a relationship between parent-reported anxiety and cardiac reactivity was not found in any group. The relationship between anxiety and cardiac activity is inconsistent across ASD and FXS, with some evidence suggesting that the interplay between anxiety, ASD severity, and cognitive level makes it difficult to isolate the impact of anxiety alone on autonomic functioning (2, 59). Anxiety in young children, especially those with developmental delays, is very difficult to accurately distinguish from other behavioral difficulties when present (56, 60–62). The reliance on parent reports for interpreting pediatric anxiety is limiting, as many of these measures are designed for neurotypical children with classic presentations of anxiety. Previous research in anxiety in ASD has suggested that higher functioning individuals show higher levels of anxiety, but these studies often rely on classic presentations of anxiety. Individuals with ASD and ID have shown increased problem behavior, elevated heart rate, and decreased RSA in anxiety-provoking situations, which is difficult to characterize as anxiety without multiple sources of data or a functional behavior assessment (62). Thus, to accurately understand and intervene in anxiety in individuals with ID, a multi-method approach that evaluates observed behaviors, physiological data, and clinical interviews is essential (62, 63).

Anxiety often becomes more distinct and easier to identify as a child ages and has a greater ability to use language to report internal feelings and experiences. Thus, anxiety in a pre-school sample with intellectual disabilities is not only challenging to measure, but may be subtle, idiosyncratic, or even absent at this stage of development. Additionally, physiological dysregulation has been posited to underlie emotion dysregulation, and thus, cardiac activity during the startle paradigm might better reflect general emotional dysregulation rather than anxiety specifically (47). Since, autonomic flexibility is associated with social functioning, language ability (3), and behavioral problems (1), the startle paradigm might capture these aspects of development rather than anxiety. In the present study, ASD severity and non-verbal cognitive ability exhibited a correlation to cardiac startle response where parent-reported anxiety did not.

Limitations

Although this is the first study to directly compare heart activity in pre-school children with ASD and FXS, some limitations should be considered. First, our groups varied in size and variability, which could mask patterns or effects in the smaller groups, particularly the FXS split sample. Additionally, while the PAS has been used in pre-school children with ID, the measure was developed for typically developing children, and thus the nuances of atypical anxiety in children with ASD and FXS (56) might be missed. Further, the PAS is a parent-reported measure, as self-report is very challenging in a young

sample with ID. While parent-report is necessary during the assessment of children, it is limited to the parent's perceptions, observations, and conclusions about their child's behavior. The addition of behavioral observations and a clinician-led anxiety interview developed for children with ASD could clarify the relationship between physiological regulation and anxiety in preschool children with neurodevelopmental disabilities.

CONCLUSIONS AND FUTURE DIRECTIONS

Individuals with neurodevelopmental disabilities often show difficulties with autonomic flexibility in response to aversive stimuli and experience emotional and behavioral dysregulation alongside physiological dysregulation (2, 64). The present study assessed cardiac startle response in children with varying risks for anxiety, ASD, and cognitive delays. The results demonstrate that physiological dysregulation begins early in childhood, during windows of time when children are particularly sensitive to intervention (65–67). Children with autonomic flexibility have better language, cognitive, and social skills (3, 23), and thus, the relationship between behavioral and physiological functioning must be considered in early interventions. Evidence suggests that incorporating physiological components, like relaxation and neurofeedback, with learning social and cognitive skills can lead to more skill acquisition by individuals with ASD (68–71). Therefore, interventions addressing physiological regulation in response to environmental stressors paired with skill-building components can prime the child to be more receptive to interventions targeting sleep, attention, and learning.

In order to ascertain the nuances of physiological regulation and behavioral problems in young children with neurodevelopmental disabilities, future work should incorporate behavioral observations alongside physiological measures. Additionally, integrating measures of sensory behaviors and emotion regulation could elicit insight into the interplay of behavioral, physiological, and neurological factors impacting child development. Further, studies in older and higher-functioning samples of ASD have found differences in physiological response when groups are divided into subgroups of high and low anxiety (27, 72). Thus, distinguishing clinical groups by anxiety symptoms might delineate the relationship between high anxiety symptoms and physiological reactivity. Lastly, including clinical interviews of anxiety that target both traditional and atypical presentations in young children are important to clarify the role of anxiety in high-risk populations. Overall, a richer understanding of the complex relationship of physiological markers and behavior difficulties in young children with neurodevelopmental disabilities is a critical step in developing and refining appropriate interventions for early childhood.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by University of South Carolina Institutional Review Board. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

JR served as principal investigator of the parent study. JE and JR conceptualized the paper and finalized the paper. JE, JR, and AH drafted the manuscript. EW consulted on the analytic plan and reviewed the section Results. KS was instrumental in the collection and processing of cardiac recordings. All authors provided feedback to early drafts.

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REFERENCES

1. Beauchaine T. Vagal tone, development, and Gray's motivational theory: toward an integrated model of autonomic nervous system functioning in psychopathology. *Dev Psychopathol.* (2001) 13:183–214. doi: 10.1017/S0954579401002012
2. Klusek J, Roberts JE, Losh M. Cardiac autonomic regulation in autism and Fragile X syndrome: a review. *Psychol Bull.* (2015) 141:141–75. doi: 10.1037/a0038237
3. Patriquin MA, Scarpa A, Friedman BH, Porges SW. Respiratory sinus arrhythmia: a marker for positive social functioning and receptive language skills in children with autism spectrum disorders. *Dev Psychobiol.* (2013) 55:101–12. doi: 10.1002/dev.21002
4. Schmitz J, Krämer M, Tuschen-Caffier B, Heinrichs N, Blechert J. Restricted autonomic flexibility in children with social phobia: autonomic nervous system in children. *J Child Psychol Psychiatry.* (2011) 52:1203–11. doi: 10.1111/j.1469-7610.2011.02417.x
5. Bakker MJ, Tijssen MAJ, van der Meer JN, Koelman JHTM, Boer F. Increased whole-body auditory startle reflex and autonomic reactivity in children with anxiety disorders. *J Psychiatry Neurosci.* (2009) 34:314–22.
6. Levine JC, Fleming R, Piedmont JI, Cain SM, Chen W-J. Heart rate variability and generalized anxiety disorder during laboratory-induced worry and aversive imagery. *J Affect Disord.* (2016) 205:207–15. doi: 10.1016/j.jad.2016.07.019
7. Porges SW. Vagal tone: a physiologic marker of stress vulnerability. *Pediatrics.* (1992) 90:498–504.
8. Thayer JF, Friedman BH, Borkovec TD. Autonomic characteristics of generalized anxiety disorder and worry. *Biol Psychiatry.* (1996) 39:255–66.
9. Davis M. Neural systems involved in fear and anxiety measured with fear-potentiated startle. *Am Psychol.* (2006) 61:741–56. doi: 10.1037/0003-066X.61.8.741
10. Porges SW. Cardiac vagal tone: a physiological index of stress. *Neurosci Biobehav Rev.* (1995) 19:225–33.

11. Gorka SM, Nelson BD, Sarapas C, Campbell M, Lewis GF, Bishop JR, et al. Relation between respiratory sinus arrythymia and startle response during predictable and unpredictable threat. *J Psychophysiol.* (2013) 27:95–104. doi: 10.1027/0269-8803/a000091
12. Paniccia M, Paniccia D, Thomas S, Taha T, Reed N. Clinical and non-clinical depression and anxiety in young people: a scoping review on heart rate variability. *Auton Neurosci.* (2017) 208:1–14. doi: 10.1016/j.autneu.2017.08.008
13. American Psychiatric Publishing. *Diagnostic and Statistical Manual of Mental Disorders (dsm-5(r)). 5th ed.* Washington, DC: APA Publishing (2013).
14. Maenner MJ, Shaw KA, Baio J, EdS1, Washington A, Patrick M, et al. Prevalence of Autism spectrum disorder among children aged 8 years - Autism and Developmental Disabilities Monitoring Network, 11 sites, United States, 2016. *MMWR Surveill Summ*. (2020) 69:1–12. doi: 10.15585/MMWR.SS6904A1
15. van Steensel FJA, Bögels SM, Perrin S. Anxiety disorders in children and adolescents with autistic spectrum disorders: a meta-analysis. *Clin Child Fam Psychol Rev*. (2011) 14:302–17. doi: 10.1007/s10567-011-0097-0
16. White SW, Oswald D, Ollendick T, Scahill L. Anxiety in children and adolescents with autism spectrum disorders. *Clin Psychol Rev*. (2009) 29:216–29. doi: 10.1016/j.cpr.2009.01.003
17. Cohen IL, A. theoretical analysis of the role of hyperarousal in the learning and behavior of fragile X males. *Ment Retard Dev Disabil Res Rev*. (1995) 1:286–91.
18. Oostra BA, Willemsen R. A fragile balance: FMR1 expression levels. *Hum Mol Genet*. (2003) 12(suppl 2):R249–57. doi: 10.1093/hmg/ddg298
19. Cordeiro L, Ballinger E, Hagerman R, Hessl D. Clinical assessment of DSM-IV anxiety disorders in fragile X syndrome: prevalence and characterization. *J Neurodev Disord*. (2011) 3:57–67. doi: 10.1007/s11689-010-9067-y
20. Ezell J, Hogan A, Fairchild A, Hills K, Klusek J, Abbeduto L, et al. Prevalence and predictors of anxiety disorders in adolescent and adult males with autism spectrum disorder and fragile X syndrome. *J Autism Dev Disord.* (2019) 49:1131–41. doi: 10.1007/s10803-018-3804-6
21. Abbeduto L, Thurman AJ, McDuffie A, Klusek J, Feigles RT, Ted Brown W, et al. ASD comorbidity in fragile X syndrome: Symptom profile and predictors of symptom severity in adolescent and young adult males. *J Autism Dev Disord.* (2019) 49:960–77. doi: 10.1007/s10803-018-3796-2
22. Roberts JE, Bradshaw J, Will E, Hogan AL, McQuillin S, Hills K. Emergence and rate of autism in fragile X syndrome across the first years of life. *Dev Psychopathol.* (2020) 32:1335–52. doi: 10.1017/S0954579420000942
23. Patriquin MA, Hartwig EM, Friedman BH, Porges SW, Scarpa A. Autonomic response in autism spectrum disorder: relationship to social and cognitive functioning. *Biol Psychol.* (2019) 145:185–97. doi: 10.1016/j.biopsycho.2019.05.004
24. Dijkhuis RR, Ziermans T, van Rijn S, Staal W, Swaab H. Emotional arousal during social stress in young adults with autism: Insights from heart rate, heart rate variability and self-report. *J Autism Dev Disord.* (2019) 49:2524–35. doi: 10.1007/s10803-019-04000-5
25. Hollocks MJ, Howlin P, Papadopoulos AS, Khondoker M, Simonoff E. Differences in HPA-axis and heart rate responsiveness to psychosocial stress in children with autism spectrum disorders with and without co-morbid anxiety. *Psychoneuroendocrinology.* (2014) 46:32–45. doi: 10.1016/j.psyneuen.2014.04.004
26. Corbett BA, Muscatello RA, Baldinger C. Comparing stress and arousal systems in response to different social contexts in children with ASD. *Biol Psychol.* (2019) 140:119–30. doi: 10.1016/j.biopsycho.2018.12.010
27. Panju S, Brian J, Dupuis A, Anagnostou E, Kushki A. Atypical sympathetic arousal in children with autism spectrum disorder and its association with anxiety symptomatology. *Mol Autism.* (2015) 6:64. doi: 10.1186/s13229-015-0057-5
28. Edmiston EK, Jones RM, Corbett BA. Physiological response to social evaluative threat in adolescents with Autism Spectrum Disorder. *J Autism Dev Disord.* (2016) 46:2992–3005. doi: 10.1007/s10803-016-2842-1
29. Roberts JE, Tonnsen B, Robinson A, Shinkareva SV. Heart activity and autistic behavior in infants and toddlers with fragile X syndrome. *Am J Intellect Dev Disabil.* (2012) 117:90–102. doi: 10.1352/1944-7558-117.2.90
30. Tonnsen BL, Malone PS, Hatton DD, Roberts JE. Early negative affect predicts anxiety, not autism, in preschool boys with fragile X syndrome. *J Abnorm Child Psychol.* (2013) 41:267–80. doi: 10.1007/s10802-012-9671-2
31. Roberts JE, Long ACJ, McCary LM, Quady AN, Rose BS, Widrick D, et al. Cardiovascular and behavioral response to auditory stimuli in boys with fragile X syndrome. *J Pediatr Psychol.* (2013) 38:276–84. doi: 10.1093/jpepsy/jss114
32. Boccia ML, Roberts JE. Behavior and autonomic nervous system function assessed via heart period measures: the case of hyperarousal in boys with fragile X syndrome. *Behav Res Methods Instrum Comput.* (2000) 32:5–10. doi: 10.3758/BF03200783
33. Hernandez RN, Feinberg RL, Vaurio R, Passanante NM, Thompson RE, Kaufmann WE. Autism spectrum disorder in fragile X syndrome: a longitudinal evaluation. *Am J Med Genet A*. (2009) 149A:1125–37. doi: 10.1002/ajmg.a.32848
34. Mian ND, Carter AS, Pine DS, Wakschlag LS, Briggs-Gowan MJ. Development of a novel observational measure for anxiety in young children: the anxiety dimensional observation scale. *J Child Psychol Psychiatry.* (2015) 56:1017–25. doi: 10.1111/jcpp.12407
35. Porges SW, Bohrer RE. The analysis of periodic processes in psychophysiological research. In: Cacioppo JT, Tassinary LG, editors. *Principles of Psychophysiology: Physical, Social, and Inferential Elements*. Cambridge: Cambridge University Press (1990). p. 708–53.
36. Lord C, Rutter M, DiLavore P, Risi S, Gotham K, Bishop S. *Autism Diagnostic Observation. Schedule—2nd edition (ADOS-2).* Los Angeles, CA: Western Psychological Corporation (2012).
37. Spence SH, Rapee R, McDonald C, Ingram M. The structure of anxiety symptoms among preschoolers. *Behav Res Ther.* (2001) 39:1293–316. doi: 10.1016/S0005-7967(00)00098-X
38. Carruthers S, Kent R, Hollocks MJ, Simonoff E. Brief report: testing the psychometric properties of the Spence Children's Anxiety Scale (SCAS) and the Screen for child anxiety related emotional disorders (SCARED) in autism spectrum disorder. *J Autism Dev Disord.* (2020) 50:2625–32. doi: 10.1007/s10803-018-3774-8
39. Wigham S, Rodgers J, South M, McConachie H, Freeston M. The interplay between sensory processing abnormalities, intolerance of uncertainty, anxiety and restricted and repetitive behaviours in autism spectrum disorder. *J Autism Dev Disord.* (2015) 45:943–52. doi: 10.1007/s10803-014-2248-x
40. Zainal H, Magiati I, Tan JW-L, Sung M, Fung DSS, Howlin P, et al. preliminary investigation of the Spence Children's Anxiety Parent Scale as a screening tool for anxiety in young people with autism spectrum disorders. *J Autism Dev Disord.* (2014) 44:1982–94. doi: 10.1007/s10803-014-2075-0
41. Mullen E. *Mullen Scales of Early Learning*. Circle Pines, MN: AGS. (1995).
42. Lord C, Risi S, Pickles A. Trajectory of language development in autistic spectrum disorders. In: Rice ML, Warren SF, editors. *Developmental Language Disorders: From Phenotypes to Etiologies*. New York, NY: Psychology Press (2004). p. 7–29. doi: 10.4324/9781410610881
43. Akshoomoff N. Use of the Mullen Scales of Early Learning for the assessment of young children with Autism Spectrum Disorders. *Child Neuropsychol.* (2006) 12:269–77. doi: 10.1080/09297040500473714
44. Stephens RL, Langworthy B, Short SJ, Goldman BD, Girault JB, Fine JP, et al. Verbal and nonverbal predictors of executive function in early childhood. *J Cogn Dev.* (2018) 19:182–200. doi: 10.1080/15248372.2018.1439493
45. Cohen S, Masyn K, Mastergeorge A, Hessl D. Psychophysiological responses to emotional stimuli in children and adolescents with autism and fragile X syndrome. *J Clin Child Adolesc Psychol.* (2015) 44:250–63. doi: 10.1080/15374416.2013.843462
46. Garber KB, Visootsak J, Warren ST. Fragile X syndrome. *Eur J Hum Genet.* (2008) 16:666–72. doi: 10.1038/ejhg.2008.61
47. White SW, Mazefsky CA, Dichter GS, Chiu PH, Richey JA, Ollendick TH. Social-cognitive, physiological, and neural mechanisms underlying emotion regulation impairments: understanding anxiety in autism spectrum disorder. *Int J Dev Neurosci.* (2014) 39:22–36. doi: 10.1016/j.ijdevneu.2014.05.012
48. Bailey DB Jr, Hatton DD, Skinner M, Mesibov G. Autistic behavior, FMR1 protein, and developmental trajectories in young males with fragile X syndrome. *J Autism Dev Disord.* (2001) 31:165–74. doi: 10.1023/A:1010747131386
49. Caravella KE, Roberts JE. Adaptive skill trajectories in infants with fragile X syndrome contrasted to typical controls and infants at high risk for autism. *Res Autism Spectr Disord.* (2017) 40:1–12. doi: 10.1016/j.rasd.2017.05.002

50. Hahn LJ, Brady NC, Warren SF, Fleming KK. Do children with fragile X syndrome show declines or plateaus in adaptive behavior? *Am J Intellect Dev Disabil.* (2015) 120:412–32. doi: 10.1352/1944-7558-120.5.412
51. Hogan AL, Caravella KE, Ezell J, Rague L, Hills K, Roberts JE. Autism spectrum disorder symptoms in infants with fragile X syndrome: a prospective case series. *J Autism Dev Disord.* (2017) 47:1628–44. doi: 10.1007/s10803-017-3081-9
52. Kaufmann WE, Cortell R, Kau ASM, Bukelis I, Tierney E, Gray RM, et al. Autism spectrum disorder in fragile X syndrome: communication, social interaction, and specific behaviors. *Am J Med Genet A.* (2004) 129A:225–34. doi: 10.1002/ajmg.a.30229
53. Klusek J, Martin GE, Losh M. Consistency between research and clinical diagnoses of autism among boys and girls with fragile X syndrome: Rates of autism in fragile X syndrome. *J Intellect Disabil Res.* (2014) 58:940–52. doi: 10.1111/jir.12121
54. Lydon S, Healy O, Reed P, Mulhern T, Hughes BM, Goodwin MS, et al. systematic review of physiological reactivity to stimuli in autism. *Dev Neurorehabil.* (2016) 19:335–55. doi: 10.3109/17518423.2014.971975
55. Geisler FCM, Kubiak T, Siewert K, Weber H. Cardiac vagal tone is associated with social engagement and self-regulation. *Biol Psychol.* (2013) 93:279–86. doi: 10.1016/j.biopsycho.2013.02.013
56. Kerns CM, Kendall PC, Berry L, Souders MC, Franklin ME, Schultz RT, et al. Traditional and atypical presentations of anxiety in youth with autism spectrum disorder. *J Autism Dev Disord.* (2014) 44:2851–61. doi: 10.1007/s10803-014-2141-7
57. Kleberg JL, Högström J, Nord M, Bölte S, Serlachius E, Falck-Ytter T. Autistic traits and symptoms of social anxiety are differentially related to attention to others' eyes in social anxiety disorder. *J Autism Dev Disord.* (2017) 47:3814–21. doi: 10.1007/s10803-016-2978-z
58. Plesa Skwerer D, Joseph RM, Eggleston B, Meyer SR, Tager-Flusberg H. Prevalence and correlates of psychiatric symptoms in minimally verbal children and adolescents with ASD. *Front Psychiatry.* (2019) 10:43. doi: 10.3389/fpsyt.2019.00043
59. Kushki A, Brian J, Dupuis A, Anagnostou E. Functional autonomic nervous system profile in children with autism spectrum disorder. *Mol Autism.* (2014) 5:39. doi: 10.1186/2040-2392-5-39
60. Freeman RL, Horner RH, Reichle J. Relation between heart rate and problem behaviors. *Am J Ment Retard.* (1999) 104:330–45. doi: 10.1352/0895-8017(1999)104<0330:RBHRAP>2.0.CO;2
61. Kerns CM, Kendall PC, Zickgraf H, Franklin ME, Miller J, Herrington J. Not to be overshadowed or overlooked: functional impairments associated with comorbid anxiety disorders in youth with ASD. *Behav Ther.* (2015) 46:29–39. doi: 10.1016/j.beth.2014.03.005
62. Moskowitz LJ, Mulder E, Walsh CE, McLaughlin DM, Zarcone JR, Proudfit GH, et al. A multimethod assessment of anxiety and problem behavior in children with autism spectrum disorders and intellectual disability. *Am J Intellect Dev Disabil.* (2013) 118:419–34. doi: 10.1352/1944.7558.118.6.419
63. Moskowitz LJ, Walsh CE, Mulder E, McLaughlin DM, Hajcak G, Carr EG, et al. Intervention for anxiety and problem behavior in children with autism spectrum disorder and intellectual disability. *J Autism Dev Disord.* (2017) 47:3930–48. doi: 10.1007/s10803-017-3070-z
64. Kootz JP, Marinelli B, Cohen DJ. Modulation of response to environmental stimulation in autistic children. *J Autism Dev Disord.* (1982) 12:185–93. doi: 10.1007/BF01531308
65. Dawson G, Burner K. Behavioral interventions in children and adolescents with autism spectrum disorder: a review of recent findings. *Curr Opin Pediatr.* (2011) 23:616–20. doi: 10.1097/MOP.0b013e32834cf082
66. Rogers SJ. Brief report: early intervention in autism. *J Autism Dev Disord.* (1996) 26:243–6. doi: 10.1007/BF02172020
67. Rogers SJ, Vismara LA. Evidence-based comprehensive treatments for early autism. *J Clin Child Adolesc Psychol.* (2008) 37:8–38. doi: 10.1080/15374410701817808
68. Floress MT, Zoder-Martell K, Schaub R. Social skills plus relaxation training with a child with ASD in the schools. *Res Dev Disabil.* (2017) 71:200–13. doi: 10.1016/j.ridd.2017.10.012
69. LaMarca K, Gevirtz R, Lincoln AJ, Pineda JA. Facilitating neurofeedback in children with autism and intellectual impairments using TAGteach. *J Autism Dev Disord.* (2018) 48:2090–100. doi: 10.1007/s10803-018-3466-4
70. Pineda JA, Friedrich EVC, LaMarca K. Neurorehabilitation of social dysfunctions: a model-based neurofeedback approach for low and high-functioning autism. *Front Neuroeng.* (2014) 7:29. doi: 10.3389/fneng.2014.00029
71. Silverman WK, Pina AA, Viswesvaran C. Evidence-based psychosocial treatments for phobic and anxiety disorders in children and adolescents. *J Clin Child Adolesc Psychol.* (2008) 37:105–30. doi: 10.1080/15374410701817907
72. Parma V, Cellini N, Guy L, McVey AJ, Rump K, Worley J, et al. Profiles of autonomic activity in autism spectrum disorder with and without anxiety. *J Autism Dev Disord.* (2021). doi: 10.1007/s10803-020-04862-0

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Combining Lovastatin and Minocycline for the Treatment of Fragile X Syndrome: Results From the LovaMiX Clinical Trial

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Background: Limited success of previous clinical trials for Fragile X syndrome (FXS) has led researchers to consider combining different drugs to correct the pleiotropic consequences caused by the absence of the Fragile X mental retardation protein (FMRP). Here, we report the results of the LovaMiX clinical trial, the first trial for FXS combining two disease-modifying drugs, lovastatin, and minocycline, which have both shown positive effects when used independently.

Aim: The main goals of the study were to assess the safety and efficacy of a treatment combining lovastatin and minocycline for patients with FXS.

Design: Pilot Phase II open-label clinical trial. Patients with a molecular diagnostic of FXS were first randomized to receive, in two-step titration either lovastatin or minocycline for 8 weeks, followed by dual treatment with lovastatin 40 mg and minocycline 100 mg for 2 weeks. Clinical assessments were performed at the beginning, after 8 weeks of monotherapy, and at week 20 (12 weeks of combined therapy).

Outcome Measures: The primary outcome measure was the Aberrant Behavior Checklist-Community (ABC-C) global score. Secondary outcome measures included subscales of the FXS specific ABC-C (ABC-C_{FX}), the Anxiety, Depression, and Mood Scale (ADAMS), the Social Responsiveness Scale (SRS), the Behavior Rating Inventory of Executive Functions (BRIEF), and the Vineland Adaptive Behavior Scale second edition (VABS-II).

Results: Twenty-one individuals out of 22 completed the trial. There were no serious adverse events related to the use of either drugs alone or in combination, suggesting good tolerability and safety profile of the combined therapy. Significant improvement was noted on the primary outcome measure with a 40% decrease on ABC-C global score with the combined therapy. Several outcome measures also showed significance.

Conclusion: The combination of lovastatin and minocycline is safe in patients for FXS individuals and appears to improve several elements of the behavior. These results set the stage for a larger, placebo-controlled double-blind clinical trial to confirm the beneficial effects of the combined therapy.

Keywords: fragile X syndrome, clinical trial, lovastatin, minocycline, intellectual disability, autism spectrum disorder (ASD), dual treatment

INTRODUCTION

FXS is an X-linked neurodevelopmental disorder caused by a CGG trinucleotide repeat expansion at the 5′ untranslated region of the FMR1 gene leading to its methylation and its consequent silencing. This results in reduced or absent expression of the FMRP, which is essential for proper brain development and synaptic functioning (1, 2). Clinically, FXS is characterized by moderate to severe intellectual disability in males, often accompanied by aggressivity and social avoidance, while females generally display a milder and broader cognitive phenotype. The neuropsychiatric profile of FXS includes anxiety, autism spectrum disorder (ASD) and attention deficit and/or hyperactivity disorder (ADHD). Current treatments are mostly symptomatic with limited efficacy. The latter includes antidepressants, stimulants, alpha2-agonists, and antipsychotics (3). There is a crucial need to better understand FXS core pathophysiology in order to find disease-modifying interventions capable of changing the natural trajectory of FXS and significantly reduce family burden (4).

In FXS, lack of FMRP leads to the hyper-phosphorylation of extracellular signal-regulated kinase (ERK), notably in mouse brain (5), human post-mortem brain (6, 7), and human platelets (8). Several lines of evidence suggest that reducing ERK activity reverts core features of the neurological phenotype of FXS animal models, including increased protein synthesis activity (9), cortical hyperexcitability and susceptibility to audiogenic seizures (10). These abnormalities, robustly found in preclinical models, recapitulate the enhanced risk of seizures of FXS patients and the presence of cortical hyperexcitability (11–13). Moreover, a clinical trial using lovastatin, a lipid-lowering drug that inhibits the mevalonate pathway and consequently lowers ERK phosphorylation (14), has been shown to improve the behavior of individuals with FXS aged 10 to 40 years in the context of a 3-month open-label trial (15). Interestingly, the observed decrease in ABC-C global score was somehow linked to the decrease of ERK phosphorylation in platelets, suggesting a mechanistic relationship between this pathway and behavioral outcome (8). More recently, a randomized 20-week placebo-controlled trial with lovastatin (10–40 mg/day) in 30 FXS participants (10–17 years old) was carried out introducing a parent-implemented language intervention (PILI) as primary outcome measure (16). Improved in PILI was reported in both groups during the trial without significant changes in ABC-C global scores.

In parallel to ERK phosphorylation, lack of FMRP also leads to increased matrix metalloproteinase-9 (MMP-9) activity, which has been ubiquitously described in mouse brain (17), human post-mortem brain (18, 19), and human plasma (20). Minocycline is a tetracycline antibiotic, an inhibitor of MMP-9 that showed its potency in the Fmr1 ko mouse correcting dendritic spine abnormalities (17), synaptic structures (21) while improving behavioral phenotypes (17, 22). In FXS participants, 8-weeks of treatment with minocycline was shown to improve behavior during an open-label clinical trial (23). The treatment was well-tolerated although seroconversion was reported in two subjects described as increased Anti-Nuclear Antibodies (ANA) titer. More recently, a double-blind placebo-controlled trial found that minocycline given for 3 months significantly improved the Clinical Global Impressions Scale-Improvement (CGI-I) score in children with FXS (ages 3.5–16 years) (24). Interestingly, in this double-blind trial, minocycline was shown to reduce plasmatic MMP-9 activity (20) and to improve their habituation to sound (25). Minocycline is therefore a promising drug that could correct core features of FXS pathophysiology.

Several other drugs have been tested over the years but none of them has shown clear efficacy in FXS placebo-controlled clinical trials (26). For this reason, and since the absence of FMRP has pleiotropic effects, a multi-targeted approach, combining drugs impacting different signaling pathways, could be more efficient to compensate for the absence of FMRP (27). However, there is a potential risk to target distinct receptor leading to a common mechanism such as blocking mGluR receptors and stimulate GABA in order to reduce cortical hyper-excitability (28). Since lovastatin and minocycline target clear distinct pathways, and have a very high security profile while being metabolized very differently, we believe that this synergistic drug regimen will have a higher positive effect on behavior in FXS participants, making them prime candidates for such an endeavor.

Here, we conducted an open-label, clinical trial combining lovastatin and minocycline in adolescents and adults with FXS to assess the safety and effectiveness of a combined therapy. We hypothesized that the combination of lovastatin and minocycline, each targeting distinct pathways, may have synergistic effects on cognition and behavior in individuals with FXS while not having added adverse effects.

MATERIALS AND METHODS

Study Design

The study was an open-label, single center, clinical trial designed to evaluate the safety and (to some extent) the efficacy of a dual treatment lovastatin/minocycline in adolescents and adults with FXS. The study took place at the *Centre de Recherche du*

CHUS (CRCHUS), Sherbrooke, Quebec, Canada. The enrollment period was from June 2016 to May 2017 with the last participant completing the trial in November 2017. Since dual therapy brings cumulated risk of adverse effects that could arise from either lovastatin, minocycline alone or in combination, direct exposition to both treatment at the beginning of the trial was avoided in accordance with Ethics Board recommendations, particularly in the context of a substitute consent. Prior exposition to either drugs as monotherapy for 8 weeks before 12 weeks with dual therapy was chosen to reassure participants and caregivers while facilitating monitoring of arising adverse effect's origin. This unique design also allowed the direct comparison between lovastatin and minocycline during the monotherapy period. The study was approved by the Ethics and Research Board of the CRCHUS and Health Canada being conformed to the Declaration of Helsinki.

Participants Eligibility and Regimen Allocation

Males and females with: (i) a molecular diagnosis of FXS, (ii) aged between 13 and 45 years, (iii) with an age-adjusted Wechsler Intelligence Scale score (Full Scale IQ) <70, (iv) who has a caregiver that spends at least 6 h per day with the participant and attends all visits, were eligible to participate. Exclusion criteria included the following: (i) >3 psychoactive drugs, (ii) changes in treatment regimen in the last 3 months, (iii) severe or unstable disease, (iv) pregnancy, (v) history of sustained muscle enzymes elevation and/or muscle pain, (vi) history of liver, kidney disease or systemic lupus erythematosus, (vii) concomitant drugs being metabolized by cytochrome P450 3A4.

The study was presented in detail to caregivers (being also legal representatives) and explained to participants with the help of a picture diagram. Written informed consents from legal representatives along with verbal assents from participants were obtained. Eligible participants were then assigned to lovastatin or minocycline group by the pharmacy of the CRCHUS on a 1:1 basis to treatment arms, minimizing differences in covariates (gender and age). Participants in the lovastatin group took lovastatin 20 mg during 4 weeks and 40 mg for 4 weeks, while participants in the minocycline group started with minocycline 50 mg and then 100 mg daily. Then, both groups received combined treatment of lovastatin 40 mg and minocycline 100 mg for 12 weeks. Visits at the research center were scheduled at baseline, week 8, 12, and 20 and phone calls monitoring were at week 4 and week 24 (4 weeks after completion of the trial).

Medication

Lovastatin tablets (20 and 40 mg) and minocycline capsules (50 and 100 mg) were obtained from Apotex (Toronto, Ontario, Canada). Caregivers were instructed that participants should take their medication orally every morning. If a participant had difficulty swallowing pills, we instructed caregivers that the minocycline capsules can be opened, and lovastatin tablets crushed if needed. Furthermore, caregivers were instructed that participants should avoid eating grapefruits as well as multivitamins or anti-acids during the study. To monitor compliance, subject diaries were given to caregivers and remaining tablets or capsules were counted (compliance = number of tablets taken/number of days between visits*100%).

Baseline Evaluation

Medical history, medication, and demographic information were collected at baseline. The designated caregiver filled out the French version of the Social Communication Questionnaire (SCQ). Full-scale IQ (FSIQ) of participants was assessed with the French version either of the Wechsler Intelligence Scale for Children Fourth Edition (WISC-IV) or the Wechsler Adult Intelligence Scale Third Edition (WAIS-III) by a qualified neuropsychologist. The treating specialist (FC) filled out the Clinical Global Impressions Scale-Severity (CGI-S) which ranges from 1 ("Normal, not at all ill") to 7 ("Among the most extremely ill patients") (29). Molecular diagnosis was assessed by Southern Blot and PCR-based genotyping and non-classic mutations were detected by array comparative genomic hybridization. Platelet content in FMRP was measured by immunoblots as previously described (30).

Outcome Measures

The primary outcome was the global score of the ABC-C (31, 32). Secondary outcomes included each of the FXS ABC-C subscales (ABC-C_{FX}) subscales (33), the ADAMS, the BRIEF, the SRS, the VABS-II, and the Test of Attentional Performance for Children (KiTAP) (34). With the exception of the KITAP, all questionnaires were filled out by the same caregiver of the participant, at baseline, week 8, 12, and 20 according to the behavior of the participant in the previous 2 weeks.

Safety

Vital signs, weight measurements, and physical examination were carried out at each visit. Blood samples were drawn from non-fasting participants at each visit to performed biochemical tests. Creatinine, creatine kinase (CK), alkaline phosphatase (ALP), bilirubin, alanine aminotransferase (ALT), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) were measured by enzymatic/spectrophotometric method (Roche Diagnostics® Modular P700) and apolipoprotein B (ApoB) by immunoturbidimetric assay (Roche Diagnostics® Cobas e501). Anti-nuclear antibodies (ANA) titer was determined by immunofluorescence. Non-HDL-Cholesterol (Non-HDL-C) was calculated as the difference between TC and HDL-C. Adverse events (AE) were systematically investigated with both open and close-ended questions at each visit and each scheduled phone call. Close-ended questions included those more frequently associated to lovastatin (muscle and joint pain) and minocycline treatment (teeth or skin coloration). The Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0 (35) was applied to report AE and their severity, ranging from 1 ("Mild") to 5 ("Death related AE"). A difference in ALP, creatinine, bilirubin, ALT, CK, CT, HDL, non-HDL-C, or ApoB compared to the baseline was included in the qualitative description of AE. The Liverpool causality tool was used to determine drug causality of adverse events on a 4-point scale, ranging from "Unlikely" to "Definite" (36).

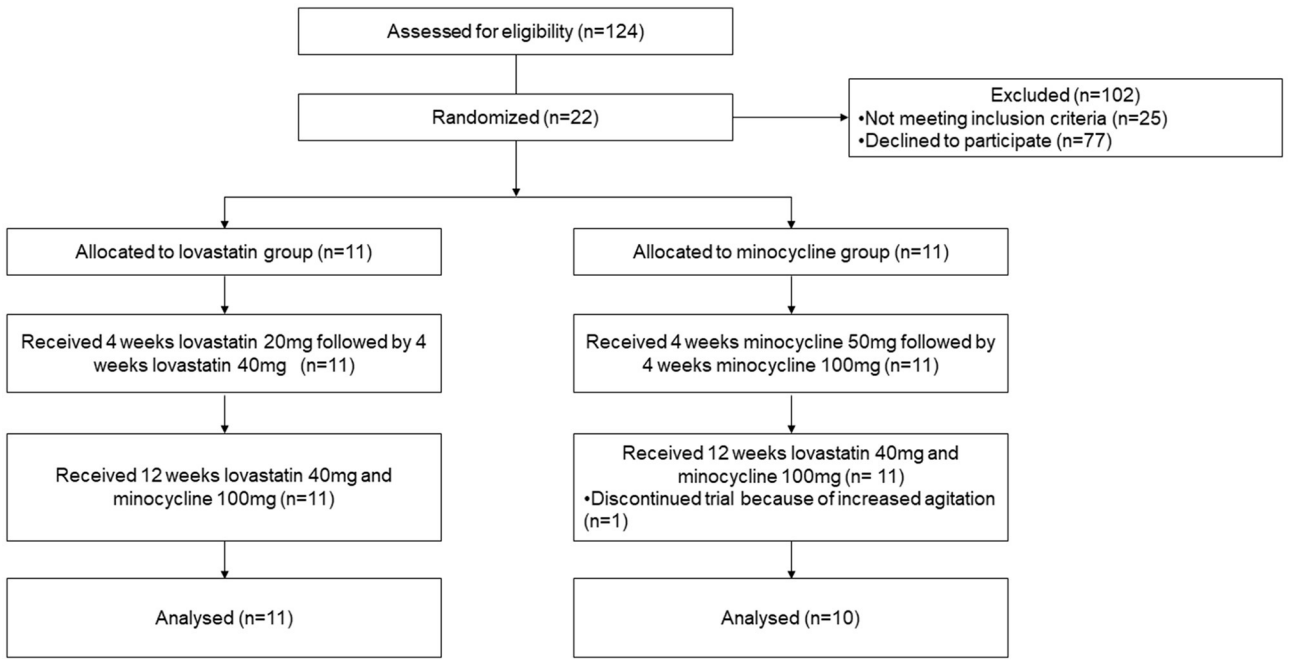

FIGURE 1 | CONsolidated Standards of Reporting Trials (CONSORT) flow diagram of subject disposition.

Statistical Analysis

A power analysis based on previous clinical trial in FXS using ABC-C global score lessening as the primary outcome recommended a sample size of 11 for each group would have at least 80% power to detect a difference in means of 19, assuming a standard deviation of differences of 19.3, using a paired *t*-test with an alpha level of 0.05 (two-sided). Data were analyzed on an intention-to-treat method, and the normality of data distribution was assessed with the Shapiro-Wilk test before applying paired Wilcoxon or paired Student's *t*-test accordingly. Following the assessment of normality with the Shapiro-Wilk test (significant at alpha 5%), differences in participants' baseline information were assessed with Wilcoxon or *t*-test for continuous variables, and with Fisher's exact test for categorical variables. Similarly, normality was assessed before applying paired Wilcoxon or paired Student's *t*-test for the differences in the primary (ABC-C_{FX} Global Score) and all secondary outcomes. To increase sensitivity for the BRIEF questionnaire, questions 21, 24, 38, and 72 and the Plan/Organize subdomain were excluded from the analysis due to too many questions being non-applicable to FXS patients. For all questionnaires, missing answers were treated according to the provider's booklet. If an answer was missing on a questionnaire for a specific participant, the specific related question was eliminated in the same way on all the other questionnaires of the participant. Adverse events were assessed with descriptive statistics. Due to the exploratory nature of the trial, we report the uncorrected *p*-values. Statistical analyses were performed with R software version 3.3.3 and GraphPad Prism version 9.2.0.

RESULTS

Participants

One hundred and twenty-four patients were contacted either directly or by e-mail to participate in the study (**Figure 1**). Of these, 77 patients declined to participate and 25 were not eligible. Recruited participants were randomly allocated to either lovastatin or minocycline group (11 per group). Only one participant out of 22 did not complete the trial. The latter was withdrawn from the study by the caregiver because of increased agitation while on the combined treatment. No statistically significant difference was found between baseline demographic information collected from both groups (**Table 1**). Of note, one female who had a deletion in the FMR1 gene instead of the classic mutation was included in the study. Noticeably, more than half of our participants were not taking any psychoactive medication before entering the study.

Compliance, Safety, and Adverse Events

Overall, mean compliance to medication for mono- and combined therapy was higher than 97% (**Table 2**). Adverse events occurring during the trial were of mild or moderate severity except for one participant who had a severe increase in ALT (>5.0 upper limit normal (ULN)) during the combined treatment period (**Table 2**). For all AE, drug-related causality ranged from unlikely to probable with none being definite. More adverse events occurred during the combined treatment phase than during monotherapy. Intriguingly, headache was again reported in few participants during lovastatin treatment as noted previously (15). Regarding biochemical tests, CK elevation

TABLE 1 | Baseline characteristics of participants.

Baseline characteristic	Lovastatin group ($n = 11$)	Minocycline group ($n = 11$)
Age	26 (19.5–26.5)	22 (19.5–25.5)
Gender—no. (%)		
Male	10 (90.91%)	9 (81.82%)
Female	1 (9.09%)	2 (18.18%)
Ethnicity—no. (%)		
Caucasian	10 (90.91%)	11 (100%)
African	1 (9.09%)	0 (0%)
Mutation type—no. (%)		
Full mutation	10 (90.91%)	10 (90.91%)
Male mosaic	1 (9.09%)	0 (0%)
Deletion	0 (0%)	1 (9.09%)
FMRP[†] (pg/10^6 platelets)	0 (0–8.2)	0 (0–5.5)
Positive ANA[‡]–no. (%)	1 (0.09%)	4 (36.36%)
FSIQ	45 (41–51.5)	48 (42–56)
SCQ score	14 (11–16.5)	15 (8–16.5)
CGI-S median (range)	4 (4–5)	4 (4–5)
Living Setting—no. (%)		
Home with family	11 (100%)	11 (100%)
Number of concomitant psychoactive medication—no. participant (%)		
None	7 (63.64%)	6 (54.55%)
One	3 (27.27%)	2 (18.18%)
Two	1 (9.09%)	0 (0%)
Three	0 (0%)	3 (27.27%)
Type of medication—no. (%)		
Antidepressant	2 (40%)	4 (36.36%)
Stimulant	2 (40%)	1 (9.09%)
Alpha2-adrenergic agonist	0 (0%)	1 (9.09%)
Antipsychotic	1 (20%)	5 (45.45%)

Data are presented as median (interquartile range) unless otherwise specified. ANA, anti-nuclear antibodies; FSIQ, full scale intellectual quotient; SCQ, social communication questionnaire; CGI-S, clinical global impressions scale-severity.

[†] *Quantified by western blot.*

[‡] *Cut-off titer at 1:80.*

was the most reported AE after 20 weeks of treatment, a well-known effect of statins. However, there was no myalgia reported by the participants. As expected, lovastatin lowered mean TC, HDL-C, ApoB of the lovastatin but minocycline groups (**Table 3**). In order to carefully monitor seroconversion, ANA titer was determined at baseline and during the course of the study. Unexpectedly, a few participants had already a positive (ANA) at baseline (**Table 1**) but no seroconversion or significant increase in ANA occurred during the trial (data not shown).

Efficacy Analyses

We observed a 40% improvement in the ABC-C Global Score, our primary outcome, after 20 weeks of treatment with both drugs when all participants of each arm are combined (**Table 4**). Several subscales were also improved either in ABC-C_{FX}, ADAMS, SRS, and BRIEF. There was also improvement in some VABS scores and in "errors with distractor" task of the KiTAP (**Supplementary Table 1**). When each group was taken separately, much less outcome measures remained statistically significant, an effect mostly attributable to smaller sample size. Nevertheless, the lovastatin but not the minocycline group showed improvement in SRS total score (**Table 4**), an effect already significant after 8 weeks of treatment with lovastatin alone (**Supplementary Table 2**).

Compared Efficacy Lovastatin/Minocycline Monotherapy

The distinctive design of our study allowed us to study not only the effect of the combined treatment but also to measure and compare the monotherapy effect of lovastatin and minocycline for 8 weeks. Although improvements were noted in most scales, they were rarely significant (**Supplementary Table 1**). The combination of small sample size (11) and a short duration of treatment (8 weeks) could explain the latter. Noteworthy, as mentioned previously, SRS seems clearly more affected by lovastatin treatment. Improvements in Inappropriate Speech (ABC-C_{FX}) and ADAMS was observed for minocycline but not lovastatin.

DISCUSSION

FXS remains a neurodevelopmental condition resulting from various alterations in absence of FMRP. The use of several medications with additive effects may be the key to a successful disease-modifying treatment (37). To our knowledge, this is the first trial assessing the safety and efficacy of a combined disease-modifying add on treatment in FXS individuals. Lovastatin and minocycline target specific pathways affected by the absence of FMRP and have shown promising effect in previous clinical trials (15, 23). These were specifically chosen because they both have a very good long-term safety profile for either treating hypercholesterolemia or acne vulgaris, respectively (38).

Security

In fact, as previously reported in monotherapy trials (15, 16, 23, 24), most adverse events following lovastatin or minocycline administration were mild, self-limited, and poorly related to treatment with the exception of CK elevation. A high percentage of participants had an initial positive ANA titer (22.7%), which is not clinically significant in absence of symptoms. More importantly, no seroconversion was observed even though half of participants were exposed to minocycline for 20 weeks. Although more adverse events occured during the combined therapy period, the treatment was very well-tolerated with only one participant not completing the study. Some characteristics of our trial may have contributed to minimize side effects. For instance, half of our participants were not taking any psychoactive medication on a regular basis limiting the bias of unknown drug interactions. Also, participants were adolescents or adults and there was less concern regarding permanent teeth discoloration (38). However, long-term safety of this specific combined therapy remains to be determined. In fact, the unknown long-term risk

TABLE 2 | Adverse events (AE).

Adverse events	Number of subjects ($n = 22$)	Number of events	Treatment period			Grade of adverse event*			Drug-related causality†		
			Lovastatin ($n = 11$)	Minocycline ($n = 11$)	Bitherapy ($n = 22$)	Mild	Moderate	Severe	Unlikely	Possible	Probable
Aggressivity	1	1			✓		✓				✓
Agitation	4	4	✓		✓✓✓	✓✓	✓✓		✓	✓✓	✓
ALP increase	1	1			✓	✓			✓		
ALT increase	2	2	✓		✓	✓		✓			✓✓
Anal pruritus	1	1			✓	✓					✓
Anorexia	4	4		✓	✓✓✓	✓✓✓✓				✓	✓✓✓
Arthralgia	1	1	✓			✓			✓		
Back pain	1	1	✓			✓			✓		
Blood bilirubin increase	1	1			✓		✓‡				✓
Cough	1	1	✓			✓			✓		
CK increase	5	5			✓✓✓✓✓	✓✓✓✓✓					✓✓✓✓✓
Diarrhea	3	3		✓	✓✓	✓✓✓				✓	✓✓
Dizziness	1	1		✓		✓				✓	
Dysmenorrhea	1	1			✓	✓			✓		
Dyspepsia	1	1			✓		✓			✓	
Fatigue	2	3		✓✓	✓	✓✓✓				✓✓✓	
Gastroenteritis	1	1	✓			✓			✓		
Headache	4	4	✓✓✓	✓		✓✓✓✓			✓✓✓	✓	
Hypersomnia	2	2		✓	✓	✓	✓			✓✓	
Neck pain	1	1			✓		✓		✓		
Pain in extremity	1	1	✓			✓			✓		
Palpitations	1	1		✓		✓				✓	
Panic attack	1	1		✓		✓				✓	
Pharyngitis	1	1			✓		✓		✓		
URTI	6	6	✓✓✓		✓✓✓		✓✓✓✓✓		✓✓✓✓✓		
Vomiting	2	2	✓		✓	✓	✓		✓	✓	
URTI	6	6	✓✓✓		✓✓✓		✓✓✓✓✓		✓✓✓✓✓		
CK increase	5	5			✓✓✓✓✓	✓✓✓✓✓					✓✓✓✓✓
Agitation	4	4	✓		✓✓✓	✓✓	✓✓		✓	✓✓	✓
Anorexia	4	4		✓	✓✓✓	✓✓✓✓				✓	✓✓✓
Headache	4	4	✓✓✓	✓		✓✓✓✓			✓✓✓	✓	
Diarrhea	3	3		✓	✓✓	✓✓✓				✓	✓✓
Fatigue	2	3		✓✓	✓	✓✓✓				✓✓✓	
ALT increase	2	2	✓		✓	✓		✓			✓✓
Hypersomnia	2	2		✓	✓	✓	✓			✓✓	
Vomiting	2	2	✓		✓	✓	✓		✓	✓	
Aggressivity	1	1			✓		✓				✓
ALP increase	1	1			✓	✓			✓		
Anal pruritus	1	1			✓	✓					✓
Arthralgia	1	1	✓			✓			✓		
Back pain	1	1	✓			✓			✓		
Blood bilirubin increase	1	1			✓		✓‡				✓
Cough	1	1	✓			✓			✓		
Dizziness	1	1		✓		✓				✓	
Dysmenorrhea	1	1			✓	✓			✓		
Dyspepsia	1	1			✓		✓			✓	
Gastroenteritis	1	1	✓			✓			✓		

(Continued)

TABLE 2 | Continued

Adverse events	Number of subjects ($n = 22$)	Number of events	Treatment period			Grade of adverse event*			Drug-related causality†		
			Lovastatin ($n = 11$)	Minocycline ($n = 11$)	Bitherapy ($n = 22$)	Mild	Moderate	Severe	Unlikely	Possible	Probable
Neck pain	1	1			✓		✓		✓		
Pain in extremity	1	1	✓			✓			✓		
Palpitations	1	1		✓		✓				✓	
Panic attack	1	1		✓		✓				✓	
Pharyngitis	1	1			✓		✓		✓		

ALP, alkaline phosphatase; ALT, alanine aminotransferase; CK, creatine kinase; URTI, upper respiratory tract infection.

**The Common Terminology Criteria for Adverse Events (CTCAE) Version 4.0 were used to describe AE. Severity ranges from 1 ("Mild") to 5 ("Death related AE"). ✓ represents each event. For biochemical values, the following ranges were used: mild (ALP > upper limit of normal (ULN) 2.5xULN, ALT > ULN−3.0xULN, bilirubin > ULN−1.5xULN, CK > ULN−2.5xULN), moderate (ALP > 2.5xULN−5.0xULN, ALT > 3.0xULN−5.0xULN, bilirubin > 1.5xULN−3.0xULN, CK > 2.5xULN−5.0xULN), and severe (ALP > 5.0xULN−20.0xULN, ALT > 5.0ULN−20.0xULN, bilirubin > 3.0ULN−10.0xULN, CK > 5.0xULN−10.0xULN). Only participants who increased in categories compared to baseline for the biochemical values were included as adverse events.*

† Drug-related causality was measured by the Liverpool causality tool.

‡ Baseline bilirubin value for this participant was of mild intensity.

TABLE 3 | Biochemical measurements.

Biochemical variables	Reference intervals*	Lovastatin group			Minocycline group		
		Baseline (n =11)	8 weeks ($n = 10$)	20 weeks ($n = 11$)	Baseline ($n = 11$)	8 weeks ($n = 11$)	20 weeks ($n = 10$)
CK	≤150 IU/L	84.27 ± 27.24	92.5 ± 30.27	105.27 ± 49	64.18 ± 28.91	70.82 ± 26.78	67.90 ± 21.86
Creatinine	37–110 μm/L	65.64 ± 8.97	64.8 ± 10.13	69.55 ± 7.98	58.09 ± 14.85	58.73 ± 15.26	60.20 ± 16.27
Lipid profile							
TC	3.2–5.48 mmol/L	3.58 ± 0.74	2.83 ± 0.61†	2.92 ± 0.55†	3.71 ± 0.59	3.61 ± 0.82	2.92 ± 0.48†
HDL-C	0.69–1.81 mmol/L	1.01 ± 0.16	1.06 ± 0.18	1.07 ± 0.22	1.02 ± 0.19	1.04 ± 0.24	1.08 ± 0.31
Non-HDL-C		2.57 ± 0.77	1.77 ± 0.68†	1.85 ± 0.68†	2.69 ± 0.69	2.57 ± 0.89	1.85 ± 0.64†
Apo B	0.6–1.6 g/L	0.77 ± 0.2	0.59 ± 0.17†	0.63 ± 0.17†	0.82 ± 0.23	0.78 ± 0.24	0.66 ± 0.19†
Liver profile							
ALP	35–485 IU/L	97.91 ± 74.44	96.9 ± 73.04	97 ± 67.96	119 ± 94	125.6 ± 103.2	127.2 ± 94.9
ALT	≤55 IU/L	26 ± 14.46	28.2 ± 14.53	26.73 ± 11.52	23.91 ± 17.52	31.09 ± 25.35	71.8 ± 125.4†
Bilirubin	2.8–17 μm/L	12.42 ± 8.59	13.78 ± 7.25	15.2 ± 14.19	8.89 ± 4.92	9.15 ± 5.66	11.65 ± 11.51

Data are presented as mean ± SD. CK, creatine kinase; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; Non-HDL-C, non-high-density lipoprotein cholesterol; Apo B, apolipoprotein B; ALP, alkaline phosphatase; ALT, alanine aminotransferase.

**Reference intervals are in accordance with Roche Diagnostics technical sheets.*

† $p \leq 0.01$.

of a sustained low cholesterol level and seroconversion should be carefully considered.

ABC-C Global Score and ABC-C_{FX} Subscales

Regarding our primary outcome, we obtained a similar reduction in the ABC-C global score with the bi-therapy as compared to our previous open label trial with lovastatin (15). However, no significant improvement was obtained in ABC-C_{FX} subscales with lovastatin monotherapy. The absence of improvement may be related to the combination of fewer participants (11 vs. 15) and a shorter period of treatment (8 vs. 12 weeks). Similarly, we did not observe a significant reduction in ABC-C global score with minocycline as reported in another Canadian center (23). However, our trial was performed with nearly half participants (11 vs. 20). Altogether, global score and almost all ABC-C_{FX} subscales (4 of 6) showed statistically significant improvement making this combined treatment a very promising one.

Other Outcomes

Several questionnaires were tested as secondary outcomes during the course of the trial, the majority being greatly recommended in FXS clinical trial (32). Many of them such as ADAMS, SRS, and BRIEF were not administered in previous trials with either minocycline or lovastatin monotherapy, greatly limiting comparison. Yet, many subscales of these questionnaires were improved and limited our ability to identify a better outcome measure than ABC-C global score. Also, beside SRS who appeared more specific to lovastatin

TABLE 4 | Outcome measures 0–20 weeks.

Endpoints	Lovastatin group ($n = 11$)			Minocycline group ($n = 10$)			Both groups ($n = 21$)		
	Baseline	**20 weeks**	***p***	**Baseline**	**20 weeks**	***p***	**Baseline**	**20 weeks**	***p***
ABC-C_{FX}									
Global score	48 (35–58)	33 (24–40)	0.054	41 (26–65)	24 (16–53)	0.143	48 (27–62)	29 (16–45)	**0.004**
Irritability	8 (3–10)	3 (1–9)	0.412	6.5 (1–14)	3 (1–9)	0.617	8 (3–11)	3 (1–10)	0.306
Lethargy	8 (6–14)	7 (3–8)	0.129	10.5 (6–14)	5.5 (1–11)	**0.009**	10 (5–14)	6 (2–9)	**0.005**
Stereotypy	6 (4–10)	5 (4–6)	0.191	5 (3–6)	4.5 (2–5)	0.375	5 (3–10)	5 (3–5)	0.111
Hyperactivity	7 (4–9)	3 (2–7)	**0.014**	5.5 (4–10)	4 (2–7)	0.262	6 (4–9)	3 (2–7)	**0.006**
Inappropriate speech	5 (4–7)	5 (3–6)	0.176	6.5 (4–8)	3.5 (2–8)	0.103	5 (4–5)	4 (2–4)	**0.028**
Social avoidance	8 (7–11)	5 (4–8)	**0.027**	5.5 (4–8)	4.5 (2–8)	0.406	7 (5–10)	5 (4–8)	**0.020**
ADAMS									
Total score	28 (17–40)	23 (16–29)	**0.043**	30 (14–49)	16 (10–37)	0.082	28 (17–40)	19 (12–29)	**0.008**
Manic/hyperactive behavior	5 (3–6)	3 (3–6)	0.169	6 (3–9)	4.5 (2–6)	0.152	5 (3–7)	4 (2–6)	**0.047**
Depressed mood	3 (1–4)	1 (1–4)	0.177	4.5 (1–10)	3.5 (1–6)	0.586	3 (1–6)	2 (1–4)	0.202
Social avoidance	11 (8–15)	8 (6–11)	0.076	8 (6–17)	5.5 (4–11)	0.056	9 (6–15)	7 (5–12)	**0.007**
General anxiety	5 (3–13)	4 (3–8)	0.063	6.5 (4–10)	3 (0–8)	0.078	6 (3–11)	3 (1–8)	**0.008**
Obsessive/compulsive behavior	2 (2–3)	2 (1–4)	0.440	4 (1–5)	1 (0–3)	0.172	2 (1–4)	2 (0–3)	0.074
BRIEF									
Inhibit	24 (20–28)	21 (19–26)	**0.005**	22.5 (19–27)	20.5 (17–23)	**0.021**	23 (20–27)	21 (17–23)	**<0.001**
Shift	23 (20–25)	19 (18–21)	**0.010**	20 (18–23)	18 (17–21)	**0.042**	21 (19–24)	19 (17–21)	**0.001**
Emotion control	17 (15–20)	15 (14–19)	**0.018**	14.5 (13–18)	13 (12–17)	0.513	16 (14–19)	15 (12–18)	**0.043**
Monitor	10 (9–12)	10 (9–11)	0.703	10.5 (8–13)	9 (7–12)	0.096	10 (8–12)	9 (8–11)	0.124
Working memory	24 (22–27)	23 (22–24)	0.092	21.5 (20–25)	21 (20–23)	0.181	22 (20–25)	22 (20–23)	**0.026**
Organize materials	10.5 (8–13)	9.5 (8–12)	0.943	9 (7–11)	9.5 (8–12)	0.052	9 (7–11)	9 (7–12)	0.366
Task completion	21 (18–22)	18 (16–22)	0.205	17.5 (15–19)	15.5 (13–18)	0.290	18 (15–19)	16 (13–18)	0.087
SRS									
Total raw score	163 (157–170)	149 (139–159)	**<0.001**	150 (130–174)	135 (131–149)	0.081	162 (138–171)	146 (133–159)	**<0.001**
Awareness	19 (18–21)	17 (17–21)	0.632	19 (18–20)	17 (16–19)	0.078	19 (18–21)	17 (16–19)	0.097
Cognition	30 (27–33)	27 (25–32)	**0.003**	30 (26–33)	26.5 (26–28)	0.305	30 (26–33)	27 (25–29)	**0.015**
Communication	52 (50–58)	49 (42–56)	**0.009**	45.5 (44–54)	44.5 (41–49)	0.274	51 (44–57)	47 (41–50)	**0.009**
Motivation	29 (27–31)	25 (23–29)	**0.048**	25 (23–29)	23.5 (22–27)	0.144	27 (24–30)	25 (22–28)	**0.011**
Mannerisms	32 (28–34)	27 (25–30)	**0.001**	29 (23–35)	24.5 (22–30)	0.130	30 (26–34)	26 (23–30)	**0.001**

Data are presented as median (interquartile range). P value < 0,05 are in bold. ABC-C_{FX}, aberrant behavior checklist-community adapted for FXS; ADAMS, anxiety, depression and mood scale; BRIEF-SR, behavior rating inventory of executive function-self-report version; SRS, social responsiveness scale.

treatment, we were unable to determine if lovastatin and minocycline improve differently, specific traits highlighted in those questionnaires.

Study Limitations

Our pilot trial had clear limitations. Owing to the intrinsic design of the trial (open-label), outcome measures are prone to the placebo effect and observer-expectancy bias. Also, the distinct contribution of the monotherapy and the dual therapy period on the overall effect of the 20 week treatment is difficult to delineate since the contribution of the placebo effect in each treatment period is undetermined. In fact, caregivers have clearly showed higher concerns with the addition of the second drug that could modulate the placebo effect during the dual therapy period. Nevertheless, additional exposure to lovastatin's monotherapy for 8 weeks may explain higher SRS improvement in the lovastatin group. Alternatively, the minocycline group had more participants taking 3 or more psychoactive medications that could lead to lesser SRS improvement. It remains unclear if starting both medications simultaneously for 12 weeks would have been as beneficial in terms of efficacy while being as safe for participants. Our short trial duration and greater age of participants make it easier to monitor adverse effects but less likely to obtain significant effects on behavior. Clearly, to determine the true benefit potential of this combined lovastatin-minocycline treatment, a placebo-controlled trial is warranted in younger individuals where the two drugs are taken at the very beginning of the trial.

This study, clearly showing the security of a combined treatment would certainly alleviate caregiver apprehension on adverse effects and facilitate recruitment for future trials using a combined treatment of lovastatin/minocycline. Our study also paves the way for future trials using other combined treatment that would better compensate for the absence of FMRP and

improve the natural evolution of FXS individuals and alleviate families' burden.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Comité d'éthique de la recherche du CIUSSS de l'Estrie - CHUS. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

CC conducted the trial, helped with coordination, instructed participants to perform the KiTAP, recorded clinical data, performed data analysis, and drafted the manuscript. FM-P conducted the Vineland interviews and the IQ testing. LB-L performed data analysis and drafted the manuscript. AÇ performed data analysis and conducted medical assessments. J-FL participated in the design of the study. FC conceived the study, participated in the design of the study, conducted medical assessments, and oversaw the execution of the trial. All authors participated to the data statistical analysis, contributed to the article, and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.762967/full#supplementary-material

REFERENCES

1. Verkerk AJ, Pieretti M, Sutcliffe JS, Fu YH, Kuhl DP, Pizzuti A, et al. Identification of a gene (FMR-1) containing a CGG repeat coincident with a breakpoint cluster region exhibiting length variation in fragile X syndrome. *Cell.* (1991) 65:905–14. doi: 10.1016/0092-8674(91)90397-H
2. Oberlé I, Vincent A, Abbadi N, Rousseau F, Hupkes PE, Hors-Cayla MC, et al. New polymorphism and a new chromosome breakpoint establish the physical and genetic mapping of DXS369 in the DXS98-FRAXA interval. *Am J Med Genet.* (1991) 38:336–42. doi: 10.1002/ajmg.1320380234
3. Bailey DB, Raspa M, Bishop E, Olmsted M, Mallya UG, Berry-Kravis E. Medication utilization for targeted symptoms in children and adults with fragile X syndrome: US survey. *J Dev Behav Pediatr.* (2012) 33:62–9. doi: 10.1097/DBP.0b013e318236c0e1
4. Bailey DB, Raspa M, Bishop E, Mitra D, Martin S, Wheeler A, et al. Health and economic consequences of fragile X syndrome for caregivers. *J Dev Behav Pediatr.* (2012) 33:705–12. doi: 10.1097/DBP.0b013e318272dcbc
5. Hou L, Antion MD, Hu D, Spencer CM, Paylor R, Klann E. Dynamic translational and proteasomal regulation of fragile X mental retardation protein controls mGluR-dependent long-term depression. *Neuron.* (2006) 51:441–54. doi: 10.1016/j.neuron.2006.07.005
6. Hoeffer CA, Sanchez E, Hagerman RJ, Mu Y, Nguyen DV, Wong H, et al. Altered mTOR signaling and enhanced CYFIP2 expression levels in subjects with fragile X syndrome. *Genes Brain Behav.* (2012) 11:332–41. doi: 10.1111/j.1601-183X.2012.00768.x
7. Wang X, Snape M, Klann E, Stone JG, Singh A, Petersen RB, et al. Activation of the extracellular signal-regulated kinase pathway contributes to the behavioral deficit of fragile x-syndrome. *J Neurochem.* (2012) 121:672–9. doi: 10.1111/j.1471-4159.2012.07722.x
8. Pellerin D, Çaku A, Fradet M, Bouvier P, Dubé J, Corbin F. Lovastatin corrects ERK pathway hyperactivation in fragile X syndrome: potential of platelet's signaling cascades as new outcome measures in clinical trials. *Biomarkers.* (2016) 21:497–508. doi: 10.3109/1354750X.2016.1160289
9. Muscas M, Louros SR, Osterweil EK. Lovastatin, not simvastatin, corrects core phenotypes in the fragile X mouse model. *eNeuro.* (2019) 6:1–12. doi: 10.1523/ENEURO.0097-19.2019
10. Osterweil EK, Chuang S-C, Chubykin AA, Sidorov M, Bianchi R, Wong RKS, et al. Lovastatin corrects excess protein synthesis and prevents epileptogenesis in a mouse model of fragile X syndrome. *Neuron.* (2013) 77:243–50. doi: 10.1016/j.neuron.2012.01.034
11. Knoth P, IS, Vannasing Major P, Michaud JL, Lippé S. Alterations of visual and auditory evoked potentials in fragile X syndrome. *Int J Dev Neurosci.* (2014) 36:90–7. doi: 10.1016/j.ijdevneu.2014.05.003
12. Ethridge LE, White SP, Mosconi MW, Wang J, Byerly MJ, Sweeney JA. Reduced habituation of auditory evoked potentials indicate cortical hyper-excitability in fragile X Syndrome. *Transl Psychiatry.* (2016) 6:e787. doi: 10.1038/tp.2016.48
13. Morin-Parent F, Champigny C, Lacroix A, Corbin F, Lepage J-F. Hyperexcitability and impaired intracortical inhibition in patients with fragile-X syndrome. *Transl Psychiatry.* (2019) 9:312. doi: 10.1038/s41398-019-0650-z
14. Cerezo-Guisado M, García-Román N, Garcia-Marin L, Alvarez A, Bragado M, Lorenzo M. Lovastatin inhibits the extracellular-signal-regulated kinase pathway in immortalized rat brain neuroblasts. *Biochem J.* (2007) 401:175–83. doi: 10.1042/BJ20060731
15. Çaku A, Pellerin D, Bouvier P, Riou E, Corbin F. Effect of lovastatin on behavior in children and adults with fragile X syndrome: an open-label study. *Am J Med Genet A.* (2014) 164A:2834–42. doi: 10.1002/ajmg.a.36750
16. Thurman AJ, Potter LA, Kim K, Tassone F, Banasik A, Potter SN, et al. Controlled trial of lovastatin combined with an open-label treatment of a parent-implemented language intervention in youth with fragile X syndrome. *J Neurodev Disord.* (2020) 12:12. doi: 10.1186/s11689-020-09315-4
17. Bilousova TV, Dansie L, Ngo M, Aye J, Charles JR, Ethell DW, et al. Minocycline promotes dendritic spine maturation and improves behavioural performance in the fragile X mouse model. *J Med Genet.* (2009) 46:94–102. doi: 10.1136/jmg.2008.061796
18. Gkogkas CG, Khoutorsky A, Cao R, Jafarnejad SM, Prager-Khoutorsky M, Giannakas N, et al. Pharmacogenetic inhibition of eIF4E-dependent Mmp9 mRNA translation reverses fragile X syndrome-like phenotypes. *Cell Rep.* (2014) 9:1742–55. doi: 10.1016/j.celrep.2014.10.064
19. Sidhu H, Dansie LE, Hickmott PW, Ethell DW, Ethell IM. Genetic removal of matrix metalloproteinase 9 rescues the symptoms

of fragile X syndrome in a mouse model. *J Neurosci.* (2014) 34:9867–79. doi: 10.1523/JNEUROSCI.1162-14.2014
20. Dziembowska M, Pretto DI, Janusz A, Kaczmarek L, Leigh MJ, Gabriel N, et al. High MMP-9 activity levels in fragile X syndrome are lowered by minocycline. *Am J Med Genet A.* (2013) 161A:1897–903. doi: 10.1002/ajmg.a.36023
21. Siller SS, Broadie K. Neural circuit architecture defects in a Drosophila model of Fragile X syndrome are alleviated by minocycline treatment and genetic removal of matrix metalloproteinase. *Dis Model Mech.* (2011) 4:673–85. doi: 10.1242/dmm.008045
22. Rotschafer SE, Trujillo MS, Dansie LE, Ethell IM, Razak KA. Minocycline treatment reverses ultrasonic vocalization production deficit in a mouse model of fragile X Syndrome. *Brain Res.* (2012) 1439:7–14. doi: 10.1016/j.brainres.2011.12.041
23. Paribello C, Tao L, Folino A, Berry-Kravis E, Tranfaglia M, Ethell IM, et al. Open-label add-on treatment trial of minocycline in fragile X syndrome. *BMC Neurol.* (2010) 10:91. doi: 10.1186/1471-2377-10-91
24. Leigh MJS, Nguyen DV, Mu Y, Winarni TI, Schneider A, Chechi T, et al. A randomized double-blind, placebo-controlled trial of minocycline in children and adolescents with fragile X syndrome. *J Dev Behav Pediatr.* (2013) 34:147–55. doi: 10.1097/DBP.0b013e318287cd17
25. Schneider A, Leigh MJ, Adams P, Nanakul R, Chechi T, Olichney J, et al. Electrocortical changes associated with minocycline treatment in fragile X syndrome. *J Psychopharmacol.* (2013) 27:956–63. doi: 10.1177/0269881113494105
26. Berry-Kravis EM, Lindemann L, Jønch AE, Apostol G, Bear MF, Carpenter RL, et al. Drug development for neurodevelopmental disorders: lessons learned from fragile X syndrome. *Nat Rev Drug Discov.* (2018) 17:280–99. doi: 10.1038/nrd.2017.221
27. Gross C, Hoffmann A, Bassell GJ, Berry-Kravis EM. Therapeutic strategies in fragile X syndrome: from bench to bedside and back. *Neurotherapeutics.* (2015) 12:584–608. doi: 10.1007/s13311-015-0355-9
28. Zeidler S, de Boer H, Hukema RK, Willemsen R. Combination therapy in fragile X syndrome; possibilities and pitfalls illustrated by targeting the mGluR5 and GABA pathway simultaneously. *Front Mol Neurosci.* (2017) 10:368. doi: 10.3389/fnmol.2017.00368
29. Guy W. *ECDEU Assessment Manual for Psychopharmacology*. Rockville, MD: National Institute of Mental Health Psychopharmacology Research Branch Mental Health Administration (1976). p. 217–22.
30. Lessard M, Chouiali A, Drouin R, Sébire G, Corbin F. Quantitative measurement of FMRP in blood platelets as a new screening test for fragile X syndrome. *Clin Genet.* (2012) 82:472–7. doi: 10.1111/j.1399-0004.2011.01798.x
31. Aman MG, Singh NN, Stewart AW, Field CJ. The aberrant behavior checklist: a behavior rating scale for the assessment of treatment effects. *Am J Ment Defic.* (1985) 89:485–91. doi: 10.1037/t10453-000
32. Budimirovic DB, Berry-Kravis E, Erickson CA, Hall SS, Hessl D, Reiss AL, et al. Updated report on tools to measure outcomes of clinical trials in fragile X syndrome. *J Neurodev Disord.* (2017) 9:14. doi: 10.1186/s11689-017-9193-x
33. Sansone SM, Widaman KF, Hall SS, Reiss AL, Lightbody A, Kaufmann WE, et al. Psychometric study of the aberrant behavior checklist in fragile X syndrome and implications for targeted treatment. *J Autism Dev Disord.* (2012) 42:1377–92. doi: 10.1007/s10803-011-1370-2
34. Knox A, Schneider A, Abucayan F, Hervey C, Tran C, Hessl D, et al. Feasibility, reliability, and clinical validity of the Test of Attentional Performance for Children (KiTAP) in Fragile X syndrome (FXS). *J Neurodev Disord.* (2012) 4:2. doi: 10.1186/1866-1955-4-2
35. U.S. Department of health and human services, National Institutes of Health, National Cancer Institute. *Common Terminology Criteria for Adverse Events (CTCAE)* (Bethesda, MD), (2009). p. 79.
36. Gallagher RM, Kirkham JJ, Mason JR, Bird KA, Williamson PR, Nunn AJ, et al. Development and inter-rater reliability of the Liverpool adverse drug reaction causality assessment tool. *PLoS ONE.* (2011) 6:e28096. doi: 10.1371/journal.pone.0028096
37. Gallagher A, Hallahan B. Fragile X-associated disorders: a clinical overview. *J Neurol.* (2012) 259:401–13. doi: 10.1007/s00415-011-6161-3
38. *CPS 2016 Compendium of Pharmaceuticals and Specialties*. Ottawa, ON: Canadian Pharmacists Association (2016).

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Increased Pain Symptomatology Among Females vs. Males With Fragile X-Associated Tremor/Ataxia Syndrome

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Individuals with the fragile X premutation report symptoms of chronic pain from multiple systems, have increased incidence of comorbid conditions where pain is a prominent feature, and pathophysiology that supports disrupted pain regulation, inflammation, and energy imbalance. Less is known about how pain manifests for the subpopulation of carriers that develop the motor and cognitive changes of fragile X-associated tremor and ataxia syndrome (FXTAS), and how pain may differ between men and women. We gathered data collected from 104 males and females with FXTAS related to chronic pain, comorbid conditions related to pain, and medications used for pain control to further explore the types of pain experienced and to better characterize how individuals with the fragile X premutation experience pain sensation across genders. We found that women experience significantly more pain symptoms than men, particularly allodynia (20 vs. 2.0%, $p = 0.008$), peripheral neuropathy pain (43.9 vs. 25.4%, $p = 0.0488$), migraine (43.9 vs. 14.5%, $p = 0.0008$), fibromyalgia (26.8 vs. 0%, $p = 0.0071$) and back pain (48.5 vs. 23.4%, $p = 0.008$). We found onset of peripheral neuropathy predicts the onset of ataxia ($\beta = 0.63 \pm 0.25$, $p = 0.019$) and tremor ($\beta = 0.56 \pm 0.17$, $p = 0.004$) across gender. Women also report significantly more anxiety (82.9 vs. 39.7%, $p < 0.001$), which has implications for ideal pain treatment. These pain symptoms need to be recognized in the medical history and treated appropriately, with consideration for overlapping comorbidities.

Keywords: FXTAS, pain, premutation, FMRP, fibromyalgia, neuropathy, migraine, anxiety

INTRODUCTION

The fragile X premutation is a genetic condition characterized by an increased number of trinucleotide repeats (55–200 CGG repeats) in the *FMR1* gene, which encodes for the fragile X mental retardation protein (FMRP) central for neuronal development and synaptic plasticity in the central and peripheral nervous systems. Expansion of over 200 CGG repeats leads to hypermethylation and gene silencing resulting in the fragile X full mutation and fragile X syndrome

(FXS). The premutation leads to enhanced levels of *FMR1* mRNA from 2 to 8 times normal and the excess mRNA causes RNA toxicity including the sequestration of proteins important for neuronal function, oxidative stress, and mitochondrial dysfunction that eventually can lead to the fragile X-associated tremor ataxia syndrome (FXTAS) (1).

FXTAS is a late-onset neurodegenerative condition that occurs in approximately 40% of male and 16% of female carriers. FXTAS affects cognition and movement in individuals, typically males > females over age 50, with core features including: gait ataxia, intention tremor, parkinsonism, executive function deficits, neuropathy and dysautonomia (2). Females with FXTAS typically have less severe movement symptoms (3) and cognitive decline (4), likely because their second X chromosome with normal *FMR1* expression protects the CNS. The percentage of cells with normal allele present on the active X chromosome is the activation ratio (AR) and higher AR may be protective against some manifestations of FXTAS (5). However, females with FXTAS have higher rates of autoimmune conditions and psychiatric manifestations (6, 7). Radiological criteria for the diagnosis of FXTAS include the major findings of white matter lesions in the middle cerebellar peduncles (MCP sign) or brainstem, and the following minor signs: white matter disease in splenium of corpus callosum and/or cerebrum, and moderate-severe brain atrophy (2). Among females with FXTAS there exists high radiological variability; fewer females have the MCP sign (9% in females vs. 60% in males) but have more diffuse cerebral white matter changes, including at the pons (4).

Of significant interest are the high frequency of pain complaints, as well as comorbid conditions with chronic pain that often start prior to FXTAS onset and vary by gender. Peripheral neuropathy is common in FXTAS (3) and can be a presenting feature of the syndrome (8). Musculoskeletal pain is common; back pain (9) and general muscle pain can often lead to the diagnosis of fibromyalgia (10), especially in women where the prevalence of fibromyalgia has been found as high as 43% (11). In the general population, fibromyalgia typically ranges between 2.4 and 6.8% among women (12).

The prevalence of migraine in premutation females has been found to be 54.2 vs. 26.8% in males (13), whereas the prevalence among females in US general population is reported to be 20.7 vs. 9.7% in males (14). In addition, there exists a high prevalence of coexisting conditions related to pain such as psychiatric problems that present in childhood or adulthood (7, 15, 16), sleep problems in up to a third of carriers (17, 18), and executive function deficits (19). These conditions often present prior to the diagnosis of FXTAS and are common.

Pain is generally classified by pain type- nociceptive and neuropathic pain, as well as at different levels-central, spinal cord gating, peripherally (20). Both nociceptive and neuropathic chronic pain are prevalent in individuals with FXTAS. Nociceptive pain is caused by mechanical or chemical damage to a body tissue such as skin, muscles, joints, or fascia; nociceptors signal the brain of the injury, leading to pain perception. Examples of nociceptive pain conditions include arthritis, musculoskeletal disorders, migraine, tension headache, and fibromyalgia. Neuropathic pain can be caused by direct damage to peripheral nerves such as in diabetic neuropathy or at the level of the CNS such as in a stroke, tumor, or neurodegenerative disease like Parkinson's disease or Multiple Sclerosis, which damage areas related to pain. Central sensitization describes a type of neuropathic pain that occurs when pain signals to the brain are pathologically amplified, resulting in a high level of pain stimuli experienced in response to normally non-painful touch stimuli (allodynia) as well as increased feeling of pain stimuli (hyperalgesia). In response, the threshold for sending a signal to the CNS increases resulting in limited signal (21). To compensate for diminished peripheral signal, the spinal cord greatly amplifies the signal it does receive *via* increasing synaptic efficacy of somatosensory neurons in the dorsal horn (22).

Previous research suggests that FMRP has a role in the development of nociceptive pain sensitization and chronic pain (23, 24), and is studied as a potential target for pain treatment (25). *FMR1* knockout mice that produce no FMRP show decreased neuropathic pain, protection from nociceptive sensitization (26–28) or IL-6 induced allodynia (29), and protection from pain-induced emotional sequelae such as depression (24). FMRP is hypothesized to mediate translational control over allodynia and persistent nociceptive sensitization (29). The primary pathophysiology of FXTAS is thought to be through increased mRNA production and toxicity. Despite increased mRNA production, FMRP is paradoxically decreased as CGG repeats increase, likely secondary to less efficient translation due to excessive repeats (30, 31). Because individuals with FXTAS frequently complain of chronic pain we have surveyed 104 patients with FXTAS to clarify what types of pain occur in this disorder.

MATERIALS AND METHODS

Participants

This study was conducted at the Fragile X Research and Treatment Center of the MIND Institute, University of California, Davis Medical Center, with participants in the Genotype-Phenotype Relationships in Fragile-X families data set. Participants with FXTAS were identified through families of known members affected by fragile X syndrome, referral from physicians, and *via* self-referral. All participants gave written informed consent from the UCD IRB and were categorized by the stages of FXTAS (32): stage (0) no tremor/ataxia; (1) questionable tremor and/or ataxia; (2) mild tremor and/or balance problems with minimal interference in activities of daily living (ADLs); (3) moderate tremor and/or ataxia with significant interference in ADLs; (4) severe tremor and/or ataxia requiring a cane or walker; (5) requiring daily use of wheelchair; (6) bedridden. We have eight participants who do not meet the diagnostic criteria for FXTAS (2), although all have premutation and subtle neurological symptoms apart from one patient who has the MCP sign without tremor or ataxia, a phenomenon previously reported in five males (33). These patients likely represent the most subtle end of the spectrum involving neurological deficits in the premutation and are included for comparison purposes. Participants who reported medical conditions such as a history

of stroke, head injury, or primary language other than English were excluded from the study.

Clinical Assessment and Molecular Analysis

Participants underwent medical and neurological evaluation to assess FXTAS diagnosis, stage, and *FMR1* molecular studies for CGG repeat size and mRNA level. A medical history was taken at the time of visit to document FXTAS phenotype and symptoms including tremor and ataxia in addition to age of onset and severity, current and past medication use, self-reported anxiety and depression, musculoskeletal and autoimmune problems such as osteopenia, osteoporosis, osteoarthritis, rheumatoid arthritis, fibromyalgia, lupus, ANA positivity, and other immunological diseases. We collected data on restless legs syndrome, severe cramps, disc/spine problems, and muscle pain. Migraine with and without aura, age of onset, and frequency was collected along with symptoms of neuropathy, allodynia, back pain, and chronic pain in general. Activation ratio was measured using ratios of signal intensity with Southern blot as previously described (34). *FMR1* CGG repeat allele length was quantified using a combination of both PCR and Southern blot analysis as previously described (35).

Medication Grouping

Pain medication categories were separated into non-overlapping categories of opiate analgesics, non-opiate analgesics, cannabinoids, anesthetics, antimigraine, nerve pain, and muscle pain/relaxation. Non-opiate analgesics included non-steroidal anti-inflammatory drugs (NSAIDs) and acetaminophen. Anesthetics included lidocaine. The category of antimigraine medications did not overlap with other medications categories and contained triptans, topiramate, and botulinum injections. Nerve pain medications were based on first line treatments and include tricyclics, serotonin and norepinephrine reuptake inhibitors (SNRIs), and anti-epileptic drugs (AEDs) including gabapentin, pregabalin, and carbamazepine. Muscle pain/relaxation medications included antispasticity and antispasmodic agents such as baclofen, tizanidine, and cyclobenzaprine.

Statistical Analysis

Statistical analyses of data were performed with an open-source R software. Results were expressed as mean ± standard deviation (or error) of mean for continuous variables and proportion (%) for categorical variables. For quantitative variables, group differences in means or medians were determined by *t*-test, analysis of variance (ANOVA), or Kruskal-Wallis test as appropriate. For categorical variables, proportions were compared between groups using *t*-test for proportions, chi-squared test, or Fisher's exact test as appropriate. Linear regression analysis was performed to assess associations between two variables with the main effects of the predictor and gender and their interaction term included in the linear models. Missing values were excluded from the statistical inference tests. Two-tailed $p < 0.05$ were considered statistically significant as appropriate.

RESULTS

Clinical Characteristics

The participants are 104 individuals (41 female and 63 male) with FXTAS and the premutation confirmed *via* molecular studies. No significant gender difference was found for race and ethnicity ($p = 0.513$), education level ($p = 0.199$), age ($p = 0.743$), CGG repeat size ($p = 0.266$), or FXTAS stage ($p = 0.404$). The mean (SD) age of females was 68.1 (8.8) years and age of males was 67.5 (8.0) years. Race and ethnicity are primarily white (100% female vs. 95.7% male). Of the 41 females in the study, 34 (85%) are FXTAS stage >2. Of the 63 males, 57 (90.5%) are FXTAS stage >2. The mean CGG repeat size for females is 87 (SD = 18), and the mean for males is 90 (SD = 16). Refer to **Table 1**. There is a significant difference in FXTAS diagnostic categories ($p = 0.001$) with more males diagnosed with "Definite FXTAS" than females (48.3% male vs. 9.8% female). Females are more represented in the Probable category (30.0% male vs. 58.5% female).

Age of Symptom Onset

Mean age of onset of migraine is 28.7 years (SD = 17.6), chronic pain is 51.4 years (SD = 17.3), peripheral neuropathy is 57.4 years (SD = 11.4), tremor is 58.6 years (SD = 9.2), and ataxia is 60.7 (9.4), and no gender differences were found (see **Table 1**).

The age of onset of peripheral *neuropathy* symptoms was positively associated with the age of onset of ataxia ($\beta = 0.56 \pm 0.17$, $p = 0.004$) and tremor ($\beta = 0.56 \pm 0.17$, $p = 0.004$). There were no significant associations between the onset of chronic pain and migraine with the onset of ataxia and tremor (see **Table 2** and **Figure 1**). In addition, there were no gender differences for any of those associations.

Pain Prevalence in FXTAS

There were significant gender differences in the prevalence of several symptoms. Back pain was significantly higher in females than males (48.5 vs. 23.4%, $p = 0.008$), as was migraine (43.9 vs. 14.5%, $p = 0.0008$), fibromyalgia (26.8 vs. 0%, $p = 0.0071$), thyroid problems (34.2 vs. 14.5%, $p = 0.0182$), osteoarthritis (65.9 vs. 43.5%, $p = 0.027$), allodynia (20 vs. 2.0%, $p = 0.008$), peripheral neuropathy pain (43.9 vs. 25.4%, $p = 0.0488$), and anxiety (82.9 vs. 39.7%, $p < 0.001$). No significant gender differences were found in autoimmune problems, musculoskeletal pain, or depression (see **Table 3**).

CGG Repeat and Pain

Among participants who experienced pain, there were no gender differences in CGG repeat length. Among participants who had experienced autoimmune problems, there was a significant gender difference in CGG repeat length. Females with autoimmune had significantly lower CGG repeats than that of males (76.5 ± 12.9 female vs. 105.5 ± 26.5 male, $p = 0.032$). Refer to **Supplementary Table 4**.

Musculoskeletal vs. Neuropathic Pain

Overall, the prevalence of patients with musculoskeletal pain (severe cramps + muscle pain + arthritis + back pain + disc or spine problems) was significantly higher than the prevalence

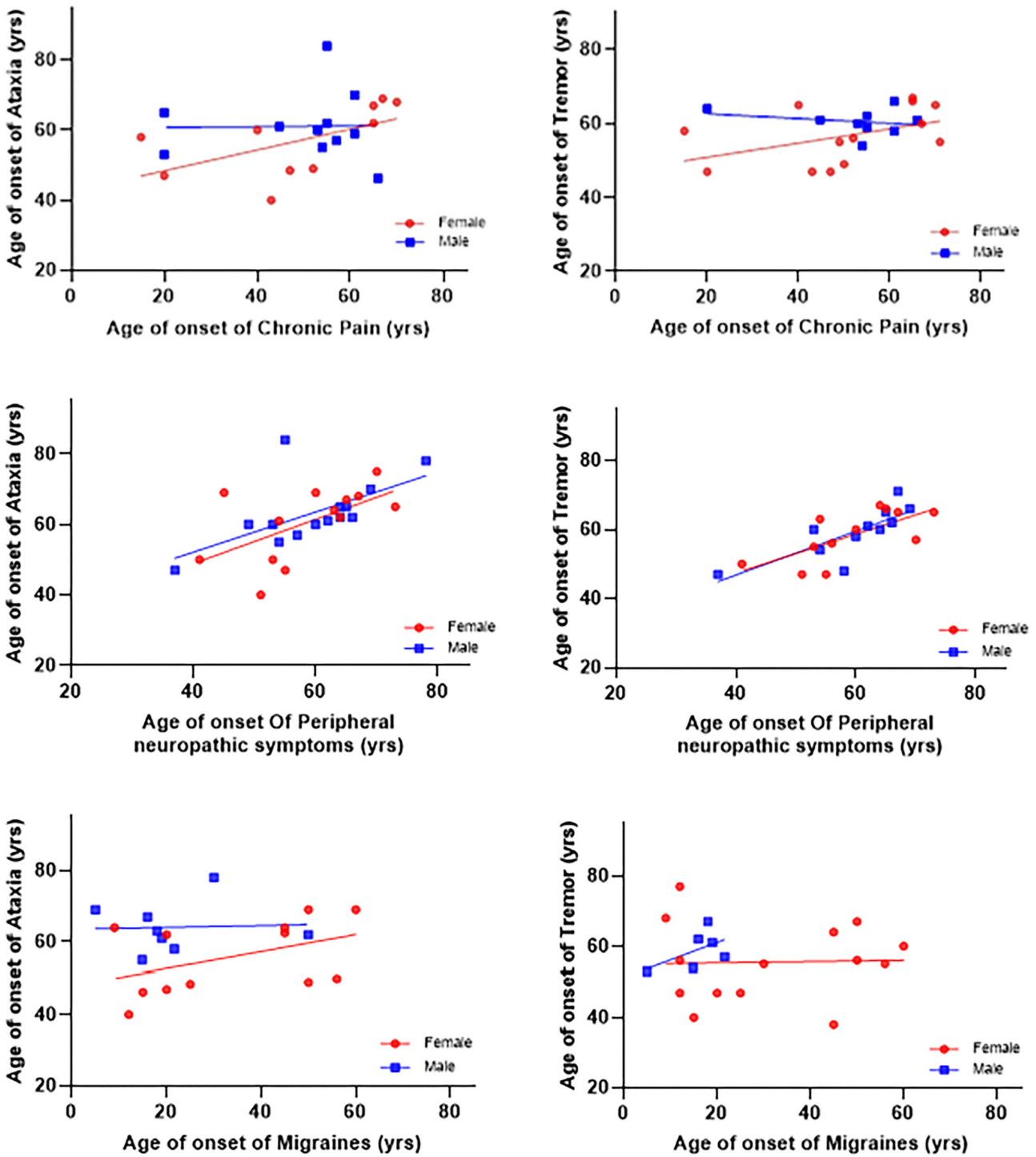

FIGURE 1 | Associations between ages of onset of symptoms and ataxia and tremor. The age of onset of peripheral *neuropathy* symptoms was positively associated with age of onset ataxia ($\beta = 0.56 \pm 0.17$, $p = 0.004$) and tremor ($\beta = 0.56 \pm 0.17$, $p = 0.004$). There were no significant associations between the onset of chronic pain and migraines with the onset of ataxia and tremor.

of patients with peripheral neuropathic pain (PNP) (88.4 vs. 33.7%, $p < 0.0001$). The difference between the proportion with neuropathic pain and the proportion with musculoskeletal pain was significant in both males and females, $p =< 0.0001$ (85.5 vs. 25.5%) and $p =< 0.0001$ (92.5 vs. 45%), respectively (see **Supplementary Table 5**).

TABLE 1 | Summary statistics [Mean ± SD or N(%)] of patient characteristics and age onset (years) of symptoms.

	Total ($N = 104$)	Female ($N = 41$)	Male ($N = 63$)	*p-value* (gender difference)
Age of visit (years)	67.8 ± 8.3	68.1 ± 8.8	67.5 ± 8.0	0.7431
FXTAS stage				
0/1	12 (11.7%)	6 (15.0%)	6 (9.5%)	0.5305
2	23 (22.3%)	10 (25.0%)	13 (20.6%)	0.6335
3	49 (47.6%)	17 (42.5%)	32 (50.8%)	0.4269
4	13 (12.6%)	4 (10.0%)	9 (14.3%)	0.7619
5	6 (5.8%)	3 (7.5%)	3 (4.8%)	0.6751
CGG repeat size	89 ± 16.4	86.7 ± 17.7	90.4 ± 15.5	0.2802
Age of onset (years)				
Tremor	58.61 ± 9.16	58.83 ± 9.36	58.44 ± 9.13	0.8591
Ataxia	60.71 ± 9.44	59.81 ± 11.82	61.33 ± 7.48	0.5011
Chronic pain	51.36 ± 17.3	52.27 ± 19.27	50.15 ± 15	0.7541
Peripheral neuropathy symptoms	57.36 ± 11.36	55.62 ± 12.92	59 ± 9.79	0.4021
Migraines	28.73 ± 17.63	32.62 ± 19.22	21.8 ± 12.49	0.1441

TABLE 2 | Associations between age onset of symptoms and age onset of ataxia and tremor.

Symptom	Ataxia				Tremor			
	Main effect		Gender difference		Main effect		Gender difference	
	β (SE)	*p-value*	β (SE)	*p-value*	β (SE)	*p-value*	β (SE)	*p-value*
Chronic pain	0.3 (0.17)	0.091	−0.29 (0.26)	0.280	0.19 (0.10)	0.061	−0.26 (0.18)	0.178
Peripheral neuropathy symptoms	0.63 (0.25)	**0.019**	−0.06 (0.34)	0.868	0.56 (0.17)	**0.004**	0.08 (0.25)	0.761
Migraines	0.24 (0.14)	0.120	−0.21 (0.29)	0.480	0.02 (0.15)	0.908	0.48 (0.81)	0.567

Bold values are p-values that have reached significance (P < 0.05) and are meant to draw the reader to the most important values.

Activation Ratio and Pain

The data does not provide evidence that activation ratio is significantly associated with either neuropathic pain or musculoskeletal pain, $p = 0.241$ and $p = 0.770$, respectively. The mean activation ratio for those with neuropathic pain is 0.52, while the mean activation ratio is 0.60 for those without neuropathic pain. The mean activation ratio for those with musculoskeletal pain is 0.57 while the mean activation ratio is 0.61 for those without musculoskeletal pain (see **Supplementary Table 6**).

Prevalence of Pain Medicine Usage

Females with FXTAS are significantly more likely to be taking any pain medication (58.5% females vs. 36.5% males, $p = 0.0271$) as well as nerve pain medication (31.7% females vs. 14.3% males, $p = 0.331$). No significant gender differences were found for other medication types. Refer to **Supplementary Table 7**.

DISCUSSION

Findings

Both men and women with FXTAS experience chronic neuropathic and musculoskeletal pain. However, women with FXTAS experience significantly more chronic pain than men and take more pain medications. It's currently unclear why women with FXTAS experience more chronic pain than men, particularly central sensitivity syndromes, neuropathic pain, and back pain. Possible mechanisms include differences in endocrinology, autoimmune conditions, connective tissue, inflammation, and neuropsychiatric differences.

Central Sensitivity

Females with FXTAS were more likely to have conditions that fit under the umbrella of central sensitivity syndromes, characterized by altered pain regulation such as fibromyalgia, allodynia, chronic pain, and chronic headaches (6). We also found that the defining symptom of central sensitization, allodynia, is significantly higher in women with FXTAS ($p = 0.008$). Sensitization occurs following intense or chronic peripheral noxious stimuli, tissue injury, or nerve damage that keeps the peripheral nervous system in a constant state of activity. Chronic pain conditions commonly known to lead to hypersensitivity such as back pain, arthritis, and fibromyalgia are significantly prevalent among women with FXTAS. Central sensitivity symptoms are heavily influenced by psychological stressors (21), which are significantly worse for females with FXTAS in our cohort. Despite the known role of FMRP in altering sensitization of pain and neuropathy in animal models, we did not find that these pain phenotypes correlated with CGG repeat length.

TABLE 3 | Prevalence [N of Yes (%)] of symptoms (Yes/No).

Symptoms	Total ($N = 104$)	Female ($N = 41$)	Male ($N = 63$)	*p-value* (gender difference)
Back pain	27 (33.8%)	16 (48.5%)	11 (23.4%)	**0.008**
Migraines	27 (26.2%)	18 (43.9%)	9 (14.5%)	**0.0008**
Fibromyalgia	11 (10.9%)	11 (26.8%)	0 (0%)	**0.0071**
Autoimmune	10 (12.3%)	6 (16.7%)	4 (8.9%)	0.2301
Thyroid problems	22 (22%)	13 (34.2%)	9 (14.5%)	**0.0182**
Osteoarthritis	54 (52.4%)	27 (65.9%)	27 (43.5%)	**0.027**
Musculoskeletal pain	86 (88.7%)	37 (92.5%)	49 (86%)	0.3077
Peripheral neuropathy pain	33 (33.0%)	18 (43.9%)	15 (25.4%)	**0.0488**
Allodynia	8 (9.5%)	7 (20.0%)	1 (2.0%)	**0.008**
Anxiety	59 (56.7%)	34 (82.9%)	25 (39.7%)	**<0.001**
Depression	26 (25.2%)	10 (24.4%)	16 (25.8%)	0.413

Bold values are p-values that have reached significance (P < 0.05) and are meant to draw the reader to the most important values.

Migraine

We found a quarter of participants (26.2%) reported experiencing migraines, with rates significantly higher among females than males (43.9% females vs. 14.5% males, $p = 0.0008$). Age of migraine onset had no bearing over age of ataxia and tremor onset. This confirms a previous study on *FMR1* premutation carriers and the prevalence of migraines (13). Author Au hypothesized that migraines in FXTAS are related to mitochondrial dysfunction leading to increased oxidative stress, which worsens with age. Several chronic pain syndromes prevalent in female *FMR1* premutation carriers are comorbid with migraine including fibromyalgia (36), allodynia (37), and chronic fatigue syndrome (36), suggesting diffuse alterations to the nociceptive nervous system. Individuals with migraines are also at an increased risk for anxiety and depression (38).

Psychiatric

We found that women with FXTAS self-reported significantly more anxiety symptoms (82.9% females vs. 39.7% males, $p = 0.001$); depressive symptoms were present in a quarter of participants (24.4% female vs. 25.8% male). This finding is consistent with previous studies that found women with FXTAS have more depression and emotional symptoms (4, 7). The relationship between pain and psychopathology is multi-directional; chronic pain is a strong predictor of both onset and persistence of comorbid psychiatric disorders in general, and vice versa, psychiatric disorders are also a powerful predictor of chronic pain persistence (39–41). Emotional states can also amplify the pain felt from central sensitivity syndromes (21). Women with FXTAS may report more chronic pain partly because that pain is viewed through a lens intensified by symptoms of anxiety, which play a role in pain perception (42). How significant a role mental health plays in FXTAS across gender may also be influenced by reporting bias, as men are less likely to disclose mental health problems than women in general (43).

Hormonal

Consistent with previous research on bone density in premutation carriers (44, 45), females were found to have more skeletal related problems such as osteopenia, osteoporosis, and osteoarthritis. With respect to pain, women with FXTAS reported significantly more back pain than men (48.5% of women vs. 23.4% of men, $p = 0.008$). Refer to **Table 3**. Post-menopausal women in the general population do tend to have higher incidence of back pain than men (46, 47), but percentages are similar between men and women in the 65–74 age group within the US (48). The cause of these gender differences is likely secondary to hormonal differences in post-menopausal women. The role estrogen plays in bone density is additionally important for women with the premutation because the mean age of natural menopause is reduced by about 5 years, from the typical age of about 51 to 46. Separate from this overall earlier menopause, 20% of carriers (49) can develop fragile X-associated primary ovarian insufficiency (FXPOI) with typically onset at 33 years (50). This directly decreases the lifetime exposure of estrogen and decreases bone mineral density, as early menopause is associated with osteoporosis (51). The significantly increased back pain among women is likely secondary to decreased bone density-related sequelae, which can have a devastating impact on women with FXTAS.

Neuropathy

More women report feeling peripheral neuropathic pain than men (43.9 vs. 25.4%, $p = 0.0488$). Women were also more likely to be taking medications that treat nerve pain (31.7 vs. 14.3%, $p = 0.0331$), however some of these medications overlap with treatment for anxiety, which was also significant among women with FXTAS. We did not find CGG repeat number correlated with nerve pain in men or women. Nerve conduction studies have shown men and women have similar sensory nerve fiber abnormalities (52), but men have additional motor nerve abnormalities in conduction velocity and latency (53). Pathogenesis of neuropathy is related to RNA toxicity causing the creation of intracellular inclusion bodies in neurons and glial cells (54) and peripheral tissues (55, 56) that causes axonal dysfunction.

Consistent with previous research that neuropathy is a presenting feature of FXTAS (8), we found the age onset of peripheral neuropathy was positively associated with the age

onset of ataxia ($\beta = 0.63 \pm 0.25$, $p = 0.019$) and tremor ($\beta = 0.56 \pm 0.17$, $p = 0.004$), with no gender difference. This further supports the recommendation to consider a FXTAS diagnosis in a patient presenting with neuropathy and a family history of intellectual disability, premature ovarian failure, autism, or movement disorder (8).

Connective Tissue

We found that musculoskeletal pain is significantly more prevalent than peripheral neuropathy pain (88.4 vs. 33.7%, $p < 0.0001$) in FXTAS. There was no significant gender difference in musculoskeletal pain (92.5 vs. 85.5%, $p = 0.3077$). Of interest, every patient that reported neuropathic pain also reported musculoskeletal pain, meaning all who experienced neuropathic pain also reported arthritis, back pain, severe cramping, or muscle pains. However, 54.7% of those with musculoskeletal pain did not have neuropathic pain (see **Supplementary Table 6**). Clinically, premutation carriers can manifest connective tissue-related features such as hyperextensible finger joints, large ears, and connective tissue dysplasia that are more subtle than those seen in FXS (57). Case report evidence including five females with premutation and Ehlers Danlos syndrome phenotype suggests there may be related commonalities in pathogenesis (58). Possible pathophysiologic mechanisms hypothesized include FMRP deficiency, mRNA toxicity, and sex effects (58). FMRP is known to regulate multiple connective tissue pathways including elastin, actin, and matrix metalloproteinase (MMP9), a class of enzymes involved in bone development, wound healing, and pathology such as arthritis and intracerebral hemorrhage (59, 60). Levels of connective tissue involvement correlate with level of FMRP depletion in FXS and premutation CGG repeats > 120 are more likely to be associated with connective tissue problems (61). Because the FXTAS population has minimal alterations to FMRP which result in altered regulation of these pathways, this mechanism could contribute to generating chronic musculoskeletal pain in FXTAS.

Autoimmune

We did not find a significant gender difference ($p = 0.2301$) in rheumatoid arthritis, systemic lupus erythematosus, or antinuclear antibodies positivity, but the prevalence for these conditions was quite low. Confirming previous research, we found FXTAS women are more likely to have fibromyalgia (26.8 vs. 0%, $p = 0.0071$) and thyroid problems (34.2 vs. 14.5%, $p = 0.0182$). Fibromyalgia is generally thought to be immune mediated (62), but its etiology remains unclear with some evidence linking it to small nerve fiber neuropathy (63) and central sensitization (64). Thyroid problems consisted almost entirely of autoimmune causes, including hypo and hyperthyroid states. Females with autoimmune conditions had significantly lower CGG repeats than that of males, likely because women are more likely to have autoimmune conditions at baseline. Autoimmune conditions and inflammation have strong links to pain and the primary pathophysiology of FXTAS involves RNA toxicity and inflammation. Inflammatory mediators from damaged cells induce pain and sensitization. Local tissue inflammation can result in hypersensitivity to pain *via* secondary hyperalgesia, where inflammatory mediators diffuse to uninjured nearby tissues. Females produce a larger proinflammatory immune response to tissue damage than males (65). Females with FXTAS may have increased chronic pain because they develop more inflammation contributing to sensitization and maintenance of pain.

Pain Treatment

Non-opiate pharmacologic therapies can be selected based on type of pain (nociceptive, neuropathic, central sensitization, and combination) or by targeting comorbid conditions.

Psychopharmacologic Overlap

Considering both psychiatric and pain symptoms of a patient is crucial as psychiatric disorders can exacerbate pain conditions and impede treatment adherence (66). Treatment of depression and anxiety in fragile X-associated neuropsychiatric disorders (FXAND) typically involves SSRIs or SNRIs (15, 67). In addition to targeting symptoms of depression and anxiety, antidepressants such as tricyclics (TCAs), SSRIs, SNRIs, and norepinephrine dopamine reuptake inhibitors (NDRIs) have evidence for use in chronic pain and are commonly used in the treatment of patients with FXTAS. Of the antidepressants, evidence suggests SNRIs and TCAs are the most effective for treating chronic neuropathic pain (68) as well as centralized pain (21). There is also strong evidence for the use of anticonvulsants pregabalin and gabapentin, and with moderate evidence for use of SSRIs (21).

We recommend avoidance of opiates to treat chronic pain associated with FXTAS. Opiates are commonly used to treat pain in the general population and FXTAS population, but anecdotal evidence suggests those on opioids can have a faster progression of their FXTAS symptoms (69). Opiates are reported to trigger white matter changes in chronic users (70, 71). *In vitro* evidence suggests premutation neurons are more vulnerable to environmental toxins than normal neurons (67). Drug and alcohol use has been reported to be increased in premutation carriers compared to controls (72, 73), which has resulted in opiate overdose (74). Long-term opiate use leads to tolerance, dependence, withdrawal symptoms, and can worsen chronic pain through development of opiate-induced hyperalgesia (75).

Topical treatments can be recommended for chronic pain such as diclofenac and ketoprofen for musculoskeletal issues (76). For osteoarthritis, topical NSAIDs, low-concentration capsaicin, or topical rubefacients. For neuropathic pain, topical lidocaine or high-concentration capsaicin can be used.

Emerging Pharmacotherapy

CBD is a compound typically derived from hemp that targets CB1, CB2 receptors and is an allosteric modulator of μ- and δ-opioid receptors. Evidence in *FMR1* knockout mice suggests CB2 receptor is necessary for protection against neuropathic pain, raising interest in targeting this receptor for treatment (77). A controlled trial examined pain in patients with multiple sclerosis, spinal cord, and other neurological conditions found pain control improved with CBD (78) in addition to two other studies that found pain relief (79, 80). CBD is also being explored as an

intervention in FXS (81) in an open-label trial (82) because of its benefits on sleep quality, anxiety (83), and cognitive impairment (84).

Curcumin is a polyphenol with anti-inflammatory properties extracted from turmeric that has shown promising preclinical results in treating FXTAS (85) but has not been studied clinically. Curcumin has a notable anti-inflammatory effect and pain relief for the treatment of general pain syndrome and osteoarthritis (86). However, more comprehensive long-term studies are needed, as many of the controlled trials are of low quality with industry funding. Sulforaphane is another dietary supplement with antioxidant/anti-inflammatory properties that was found to improve markers of oxidative stress and mitochondrial function in fibroblasts from FXTAS patients (87), but has not been studied for pain symptoms in FXTAS.

Allopregnanolone, a natural neurosteroid and positive allosteric modulator at GABA-A, has been studied as a safe therapy for neuropathic pain (88, 89), treatment for post-partum depression (90), and has been studied in an open-label trial for treatment of FXTAS in six patients with limited benefits on executive function and neuropathy symptoms (91). Allopregnanolone is a promising candidate for FXTAS because it improves mitochondrial dysfunction and acts to prevent reactive oxidative species (ROS) overproduction, a primary pathological mechanism driving FXTAS phenotype. ROS produced in mitochondria have been shown to contribute to central sensitization associated with pain (92). Allopregnanolone was able to protect neuronal cells against oxidative stress through improved mitochondrial antioxidant activity (93).

Non-pharmacologic

For a non-pharmacologic approach, counseling and therapy for chronic pain and psychiatric conditions has been helpful. The cognitive behavioral therapy approach to pain teaches patients skills to anticipate pain and divert attention to other thoughts, helping the patient better cope with pain (94). We also recommend daily exercise to stimulate neurogenesis, improve mitochondrial function, and decrease chronic pain (67). Focusing on maintaining sleep quality can have an impact on pain (95). We recommend other techniques for reducing overall stress such as biofeedback for relaxation, which can lead to improvements in various pain-related outcomes (96). Mindfulness stress reduction has evidence for reducing chronic pain and improving quality of life (97). Acupuncture has been studied in hundreds of controlled trials and appears to be effective for chronic pain, particularly back pain (98) and headaches (99).

Study Limitations

The limitations of this study include a sample bias, as the cohort consisted of participants with higher socioeconomic status and majority with white ethnicity. Data on anxiety and depression are self-reported and the symptomatology of pain is recorded through medical interviews. Future studies characterizing pain would benefit from measures of intensity. Furthermore, participants were enrolled through referral from physicians or family members and thus this cohort might not be a true representative of individuals with FXTAS, but the more affected ones. In addition, men are less likely to disclose health issues, and at more advanced FXTAS stages may not be able to assess themselves accurately.

CONCLUSION

In conclusion, our results have important clinical implications for the treatment of female premutation carriers with FXTAS who have increased back pain, neuropathy, and central sensitization related pain. Pain is a significant finding in FXTAS and should be questioned in the medical history for all premutation carriers and treated appropriately.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found at: https://redcap.ucdmc.ucdavis.edu/redcap/, CTSC#3704. Further enquiries can be directed to the corresponding author.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board at the University of California Davis. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

ES, DJ, and RH conceived of the presented idea and questions. DJ developed the idea and wrote the manuscript with support from ES, RH, KK, and MP. KK and MP performed statistical computations and created figures and tables. YM, RH, ES, AS, and DJ collected data. FT conducted molecular studies. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpsyt.2021.762915/full#supplementary-material

REFERENCES

1. Hagerman RJ, Hagerman P. Fragile X-associated tremor/ataxia syndrome—features, mechanisms and management. *Nat Rev Neurol.* (2016) 12:403–12. doi: 10.1038/nrneurol.2016.82
2. Hall DA, Birch RC, Anheim M, Jønch AE, Pintado E, O'Keefe J, et al. Emerging topics in FXTAS. *J Neurodev Disord.* (2014) 6:31. doi: 10.1186/1866-1955-6-31
3. Jacquemont S. Penetrance of the Fragile X–Associated Tremor/Ataxia Syndrome in a Premutation Carrier Population. *JAMA*. (2004) 291:460. doi: 10.1001/jama.291.4.460
4. Schneider A, Summers S, Tassone F, Seritan A, Hessl D, Hagerman P, et al. Women with fragile x–associated tremor/ataxia syndrome. *Mov Disord Clin Pract*. (2020) 7:910–919. doi: 10.1002/mdc3.13084
5. Hall DA, Robertson-Dick EE, O'Keefe JA, Hadd AG, Zhou L, Berry-Kravis E. X-inactivation in the clinical phenotype of fragile X premutation carrier sisters. *Neurol Genet.* (2016) 2:e45. doi: 10.1212/NXG.0000000000000045
6. Winarni TI, Chonchaiya W, Sumekar TA, Ashwood P, Morales GM, Tassone F, et al. Immune-mediated disorders among women carriers of fragile X premutation alleles. *Am J Med Genet Part A*. (2012) 158A:2473–2481. doi: 10.1002/ajmg.a.35569
7. Loesch DZ, Tassone F, Atkinson A, Stimpson P, Trost N, Pountney DL, et al. Differential progression of motor dysfunction between male and female fragile X premutation carriers reveals novel aspects of sex-specific neural involvement. *Front Mol Biosci*. (2021) 7:577248. doi: 10.3389/fmolb.2020.577246
8. Hagerman RJ, Coffey SM, Maselli R, Soontarapornchai K, Brunberg JA, Leehey MA, et al. Neuropathy as a presenting feature in fragile X-associated tremor/ataxia syndrome. *Am J Med Genet Part A*. (2007) 143A:2256–60. doi: 10.1002/ajmg.a.31920
9. Salcedo-Arellano MJ, Cabal-Herrera AM, Tassanakijpanich N, McLennan YA, Hagerman RJ. Ataxia as the major manifestation of fragile X-associated tremor/ataxia syndrome (FXTAS): case series. *Biomedicines*. (2020) 8:136. doi: 10.3390/biomedicines8050136
10. Leehey MA, Legg W, Tassone F, Hagerman R. Fibromyalgia in fragile X mental retardation 1 gene premutation carriers. *Rheumatology*. (2011) 50:2233–2236. doi: 10.1093/rheumatology/ker273
11. Coffey SM, Cook K, Tartaglia N, Tassone F, Nguyen DV, Pan R, et al. Expanded clinical phenotype of women with theFMR1 premutation. *Am J Med Genet Part A*. (2008) 146A:1009–1016. doi: 10.1002/ajmg.a.32060
12. Marques AP, Santo A de S. do E, Berssaneti AA, Matsutani LA, Yuan SLK. Prevalence of fibromyalgia: literature review update. *Rev Bras Reumatol.* (2017) 57:356–63. doi: 10.1016/j.rbre.2017.01.005
13. Au J, Akins R, Berkowitz-Sutherland L, Tang H-T, Chen Y, Boyd A, et al. Prevalence and risk of migraine headaches in adult fragile X premutation carriers. *Clin Genet*. (2013) 84:546–551. doi: 10.1111/cge.12109
14. Burch R, Rizzoli P, Loder E. The Prevalence and impact of migraine and severe headache in the United States: figures and trends from government health studies. *Headache.* (2018) 58:496–505. doi: 10.1111/head.13281
15. Hagerman RJ, Protic D, Rajaratnam A, Salcedo-Arellano MJ, Aydin EY, Schneider A. Fragile X-associated neuropsychiatric disorders (FXAND). *Front Psychiatry*. (2018) 9:564. doi: 10.3389/fpsyt.2018.00564
16. Bourgeois JA, Seritan AL, Casillas EM, Hessl D, Schneider A, Yang Y, et al. Lifetime prevalence of mood and anxiety disorders in fragile X premutation carriers. *J Clin Psychiatry.* (2011) 72:175–82. doi: 10.4088/JCP.09m05407blu
17. Hamlin A, Liu Y, Nguyen D V, Tassone F, Zhang L, Hagerman RJ. Sleep apnea in fragile X premutation carriers with and without FXTAS. *Am J Med Genet Part B Neuropsychiatr Genet.* (2011) 156:923–8. doi: 10.1002/ajmg.b.31237
18. Summers SM, Cogswell J, Goodrich JE, Mu Y, Nguyen DV, Brass SD, et al. Prevalence of restless legs syndrome and sleep quality in carriers of the fragile X premutation. *Clin Genet.* (2014) 86:181–4. doi: 10.1111/cge.12249
19. Hall DA, Robertson E, Shelton AL, Losh MC, Mila M, Moreno EG, et al. Update on the clinical, radiographic, and neurobehavioral manifestations in FXTAS and FMR1 premutation carriers. *Cerebellum.* (2016) 15:578–86. doi: 10.1007/s12311-016-0799-4
20. Merskey H, editor. Classification of chronic pain: Descriptions of chronic pain syndromes and definitions of pain terms. *Pain*. (1986) Suppl 3:226.
21. Dydyk AM, Givler A. *Central Pain Syndrome*. StatPearls (2021). Available online at: http://www.ncbi.nlm.nih.gov/pubmed/31971703 (accessed August 10, 2021).
22. Ji R-R, Kohno T, Moore KA, Woolf CJ. Central sensitization and LTP: do pain and memory share similar mechanisms? *Trends Neurosci.* (2003) 26:696–705. doi: 10.1016/j.tins.2003.09.017
23. Price TJ, Melemedjian OK. *Fragile X mental retardation protein (FMRP) and the Spinal Sensory System*. *Results Problems Cell Differ.* (2012). 54:41–59. doi: 10.1007/978-3-642-21649-7_4
24. Wang L, Almeida LEF, Nettleton M, Khaibullina A, Albani S, Kamimura S, et al. Altered nocifensive behavior in animal models of autism spectrum disorder: the role of the nicotinic cholinergic system. *Neuropharmacology*. (2016) 111:323–34. doi: 10.1016/j.neuropharm.2016.09.013
25. Mei X, Yang Y, Zhao J, Wang Y, Chen Q, Qian X, et al. Role of fragile X mental retardation protein in chronic pain. *Mol Pain*. (2020) 16:1744806920928619. doi: 10.1177/1744806920928619
26. Price TJ, Rashid MH, Millecamps M, Sanoja R, Entrena JM, Cervero F. Decreased Nociceptive sensitization in mice lacking the fragile X mental retardation protein: role of mGluR1/5 and mTOR. *J Neurosci.* (2007) 27:13958–13967. doi: 10.1523/JNEUROSCI.4383-07.2007
27. Price TJ, Melemedjian OK. *Fragile X Mental Retardation Protein (FMRP) and the Spinal Sensory System* (2012).
28. Cea-Del Rio CA, Nunez-Parra A, Freedman SM, Kushner JK, Alexander AL, Restrepo D, et al. Disrupted inhibitory plasticity and homeostasis in Fragile X syndrome. *Neurobiol Dis.* (2020) 142:1–12. doi: 10.1016/j.nbd.2020.104959
29. Asiedu MN, Tillu DV, Melemedjian OK, Shy A, Sanoja R, Bodell B, et al. Spinal protein kinase M underlies the maintenance mechanism of persistent nociceptive sensitization. *J Neurosci.* (2011) 31:6646–6653. doi: 10.1523/JNEUROSCI.6286-10.2011
30. Ludwig AL, Espinal GM, Pretto DI, Jamal AL, Arque G, Tassone F, et al. CNS expression of murine fragile X protein (FMRP) as a function of CGG-repeat size. *Hum Mol Genet.* (2014) 23:3228–38. doi: 10.1093/hmg/ddu032
31. Primerano B, Tassone F, Hagerman RJ, Hagerman P, Amaldi F, Bagni C. Reduced FMR1 mRNA translation efficiency in fragile X patients with premutations. *RNA.* (2002) 8:1482–8.
32. Bacalman S, Farzin F, Bourgeois JA, Cogswell J, Goodlin-Jones BL, Gane LW, et al. Psychiatric phenotype of the Fragile X-associated tremor/ataxia syndrome (FXTAS) in males. *J Clin Psychiatry*. (2006) 67:87–94. doi: 10.4088/JCP.v67n0112
33. Famula JL, McKenzie F, McLennan YA, Grigsby J, Tassone F, Hessl D, et al. Presence of middle cerebellar peduncle sign in FMR1 premutation carriers without tremor and ataxia. *Front Neurol*. (2018) 9:695. doi: 10.3389/fneur.2018.00695
34. Tassone F, Hagerman RJ, Iklé DN, Dyer PN, Lampe M, Willemsen R, et al. FMRP expression as a potential prognostic indicator in fragile X syndrome. *Am J Med Genet*. (1999) 84:250–61.
35. Filipovic-Sadic S, Sah S, Chen L, Krosting J, Sekinger E, Zhang W, et al. A novel FMR1 PCR method for the routine detection of low abundance expanded alleles and full mutations in fragile X syndrome. *Clin Chem.* (2010) 56:399–408. doi: 10.1373/clinchem.2009.136101
36. Tietjen GE, Brandes JL, Peterlin BL, Eloff A, Dafer RM, Stein MR, et al. Allodynia in migraine: association with comorbid pain conditions. *Headache.* (2009) 49:1333–44. doi: 10.1111/j.1526-4610.2009.01521.x
37. Tietjen GE, Herial NA, Hardgrove J, Utley C, White L. Migraine comorbidity constellations. *Headache.* (2007) 47:857–65. doi: 10.1111/j.1526-4610.2007.00814.x
38. Peres MFP, Mercante JPP, Tobo PR, Kamei H, Bigal ME. Anxiety and depression symptoms and migraine: a symptom-based approach research. *J Headache Pain.* (2017) 18:37. doi: 10.1186/s10194-017-0742-1
39. Uçar M, Sarp Ü, Karaaslan Ö, Gül AI, Tanik N, Arik HO. Health anxiety and depression in patients with fibromyalgia syndrome. *J Int Med Res.* (2015) 43:679–85. doi: 10.1177/0300060515587578
40. Ciaramella A, Poli P. Chronic Low Back Pain: Perception and Coping With Pain in the Presence of Psychiatric Comorbidity. *J Nerv Ment Dis.* (2015) 203:632–40. doi: 10.1097/NMD.0000000000000340

41. Gros DF, Szafranski DD, Brady KT, Back SE. Relations Between Pain, PTSD Symptoms, and Substance Use in Veterans. *Psychiatry.* (2015) 78:277–87. doi: 10.1080/00332747.2015.1069659
42. Woo AK. Depression and Anxiety in Pain. *Rev pain.* (2010) 4:8–12. doi: 10.1177/204946371000400103
43. Galdas PM, Cheater F, Marshall P. Men and health help-seeking behaviour: literature review. *J Adv Nurs.* (2005) 49:616–23. doi: 10.1111/j.1365-2648.2004.03331.x
44. Hundscheid RDL, Smits APT, Thomas CMG, Kiemeney LALM, Braat DDM. Female carriers of fragile X premutations have no increased risk for additional diseases other than premature ovarian failure. *Am J Med Genet A*. (2003) 117A:6–9. doi: 10.1002/ajmg.a.10862
45. Allen EG, Sullivan AK, Marcus M, Small C, Dominguez C, Epstein MP, et al. Examination of reproductive aging milestones among women who carry the FMR1premutation. *Hum Reprod.* (2007) 22:2142–52. doi: 10.1093/humrep/dem148
46. Wáng YXJ, Wáng J-Q, Káplár Z. Increased low back pain prevalence in females than in males after menopause age: evidences based on synthetic literature review. *Quant Imaging Med Surg.* (2016) 6:199–206. doi: 10.21037/qims.2016.04.06
47. Hoy DG, Raikoti T, Smith E, Tuzakana A, Gill T, Matikarai K, et al. Use of the global alliance for musculoskeletal health survey module for estimating the population prevalence of musculoskeletal pain: findings from the Solomon Islands. *BMC Musculoskelet Disord.* (2018) 19:292. doi: 10.1186/s12891-018-2198-0
48. QuickStats: Percentage* of adults aged ≥18 years who had lower back pain in the past 3 months,† by sex and age group—national health interview survey,§ United States, 2018. *MMWR Morb Mortal Wkly Rep*. (2020) 68:1196. doi: 10.15585/mmwr.mm685152a5
49. Sherman SL. Premature ovarian failure in the fragile X syndrome. *Am J Med Genet*. (2000) 97:189–94. doi: 10.1002/1096-8628(200023)97:3<189::AID-AJMG1036>3.0.CO;2-J
50. Hipp HS, Charen KH, Spencer JB, Allen EG, Sherman SL. Reproductive and gynecologic care of women with fragile X primary ovarian insufficiency (FXPOI). *Menopause.* (2016) 23:993–9. doi: 10.1097/GME.0000000000000658
51. Anasti JN, Kalantaridou SN, Kimzey LM, Defensor RA, Nelson LM. Bone loss in young women with karyotypically normal spontaneous premature ovarian failure. *Obstet Gynecol.* (1998) 91:12–5. doi: 10.1016/S0029-7844(97)00583-8
52. Ram S, Devapriya IA, Fenton G, Mcvay L, Nguyen DV, Tassone F, et al. Axonal neuropathy in female carriers of the fragile X premutation with fragile x-associated tremor ataxia syndrome. *Muscle Nerve*. (2015) 52:234–239. doi: 10.1002/mus.24515
53. Soontarapornchai K, Maselli R, Fenton-Farrell G, Tassone F, Hagerman PJ, Hessl D, et al. Abnormal nerve conduction features in fragile X premutation carriers. *Arch Neurol*. (2008) 65:495–498. doi: 10.1001/archneur.65.4.495
54. Greco CM, Berman RF, Martin RM, Tassone F, Schwartz PH, Chang A, et al. Neuropathology of fragile X-associated tremor/ataxia syndrome (FXTAS). *Brain.* (2006) 129:243–55. doi: 10.1093/brain/awh683
55. Hunsaker MR, Greco CM, Spath MA, Smits APT, Navarro CS, Tassone F, et al. Widespread non-central nervous system organ pathology in fragile X premutation carriers with fragile X-associated tremor/ataxia syndrome and CGG knock-in mice. *Acta Neuropathol.* (2011) 122:467–79. doi: 10.1007/s00401-011-0860-9
56. Gokden M, Al-Hinti JT, Harik SI. Peripheral nervous system pathology in fragile X tremor/ataxia syndrome (FXTAS). *Neuropathology.* (2009) 29:280–4. doi: 10.1111/j.1440-1789.2008.00948.x
57. Ramírez-Cheyne JA, Duque GA, Ayala-Zapata S, Saldarriaga-Gil W, Hagerman P, Hagerman R, et al. Fragile X syndrome and connective tissue dysregulation. *Clin Genet.* (2019) 95:262–7. doi: 10.1111/cge.13469
58. Tassanakijpanich N, McKenzie FJ, McLennan YA, Makhoul E, Tassone F, Jasoliya MJ, et al. Hypermobile ehlers-danlos syndrome (hEDS) phenotype in fragile X premutation carriers: case series. *J Med Genet*. (2021). doi: 10.1136/jmedgenet-2020-107609 [online ahead of print].
59. Vu TH, Shipley JM, Bergers G, Berger JE, Helms JA, Hanahan D, et al. MMP-9/Gelatinase B Is a Key Regulator of Growth Plate Angiogenesis and Apoptosis of Hypertrophic Chondrocytes. *Cell.* (1998) 93:411–22. doi: 10.1016/S0092-8674(00)81169-1
60. Wang J, Tsirka SE. Neuroprotection by inhibition of matrix metalloproteinases in a mouse model of intracerebral haemorrhage. *Brain.* (2005) 128:1622–33. doi: 10.1093/brain/awh489
61. Loesch DZ, Huggins RM, Bui QM, Taylor AK, Hagerman RJ. Relationship of deficits ofFMR1 gene specific protein with physical phenotype of fragile X males and females in pedigrees: A new perspective. *Am J Med Genet*. (2003) 118A:127–34. doi: 10.1002/ajmg.a.10099
62. Banfi G, Diani M, Pigatto PD, Reali E. T Cell Subpopulations in the physiopathology of fibromyalgia: evidence and perspectives. *Int J Mol Sci.* (2020) 21:1186. doi: 10.3390/ijms21041186
63. Üçeyler N, Zeller D, Kahn A-K, Kewenig S, Kittel-Schneider S, Schmid A, et al. Small fibre pathology in patients with fibromyalgia syndrome. *Brain*. (2013) 136:1857–67. doi: 10.1093/brain/awt053
64. Yunus MB. Towards a model of pathophysiology of fibromyalgia: aberrant central pain mechanisms with peripheral modulation. *J Rheumatol.* (1992) 19:846–50.
65. Rosen S, Ham B, Mogil JS. Sex differences in neuroimmunity and pain. *J Neurosci Res.* (2017) 95:500–8. doi: 10.1002/jnr.23831
66. Osterweis M, Kleinman A, Mechanic D, editors. *Pain and Disability: Clinical, Behavioral, and Public Policy Perspectives*. Washington (DC): National Academies Press (1987).
67. Polussa J, Schneider A, Hagerman R. Molecular advances leading to treatment implications for fragile X premutation carriers. *Brain Disord Ther*. (2014) 3:1000119. doi: 10.4172/2168-975X.1000119
68. Finnerup NB, Attal N, Haroutounian S, McNicol E, Baron R, Dworkin RH, et al. Pharmacotherapy for neuropathic pain in adults: a systematic review and meta-analysis. *Lancet Neurol.* (2015) 14:162–73. doi: 10.1016/S1474-4422(14)70251-0
69. Muzar Z, Adams PE, Schneider A, Hagerman RJ, Lozano R. Addictive substances may induce a rapid neurological deterioration in fragile X-associated tremor ataxia syndrome: a report of two cases. *Intractable Rare Dis Res*. (2014) 3:162–165. doi: 10.5582/irdr.2014.01023
70. Lin W-C, Chou K-H, Chen C-C, Huang C-C, Chen H-L, Lu C-H, et al. White matter abnormalities correlating with memory and depression in heroin users under methadone maintenance treatment. *PLoS ONE.* (2012) 7:e33809. doi: 10.1371/journal.pone.0033809
71. Li W, Li Q, Wang Y, Zhu J, Ye J, Yan X, et al. Methadone-induced damage to white matter integrity in methadone maintenance patients: a longitudinal self-control DTI study. *Sci Rep.* (2016) 6:19662. doi: 10.1038/srep19662
72. Kogan CS, Turk J, Hagerman RJ, Cornish KM. Impact of the fragile X mental retardation 1 (FMR1) gene premutation on neuropsychiatric functioning in adult males without fragile X-associated tremor/ataxia syndrome: a controlled study. *Am J Med Genet Part B Neuropsychiatr Genet*. (2008) 147B:859–72. doi: 10.1002/ajmg.b.30685
73. Dorn MB, Mazzacco MMM, Hagerman RJ. Behavioral and psychiatric disorders in adult male carriers of fragile X. *J Am Acad Child Adolesc Psychiatry*. (1994) 33:256–64. doi: 10.1097/00004583-199402000-00015
74. El-Deeb M, Adams P, Schneider A, Salcedo-Arellano MJ, Tassone F, Hagerman R. Fentanyl overdose in a female with the FMR1 premutation and FXTAS. *J Mol Genet*. (2018) 1:101. doi: 10.31038/JMG.1000101
75. Chu LF, Angst MS, Clark D. Opioid-induced hyperalgesia in humans. *Clin J Pain.* (2008) 24:479–96. doi: 10.1097/AJP.0b013e31816b2f43
76. Derry S, Wiffen PJ, Kalso EA, Bell RF, Aldington D, Phillips T, et al. Topical analgesics for acute and chronic pain in adults-an overview of Cochrane Reviews. *Cochrane database Syst Rev.* (2017) 5:CD008609–CD008609. doi: 10.1002/14651858.CD008609.pub2
77. Ramírez-López A, Pastor A, Torre R, La Porta C, Ozaita A, Cabañero D, et al. Role of the endocannabinoid system in a mouse model of Fragile X undergoing neuropathic pain. *Eur J Pain*. (2021) 25:1316–28. doi: 10.1002/ejp.1753
78. Wade DT, Robson P, House H, Makela P, Aram J. A preliminary controlled study to determine whether whole-plant cannabis extracts can improve intractable neurogenic symptoms. *Clin Rehabil.* (2003) 17:21–9. doi: 10.1191/0269215503cr581oa
79. Palmieri B, Laurino C, Vadalà M. Short-Term Efficacy of CBD-Enriched Hemp Oil in Girls with Dysautonomic Syndrome after Human Papillomavirus Vaccination. *Isr Med Assoc J.* (2017) 19:79–84.

80. Cuñetti L, Manzo L, Peyraube R, Arnaiz J, Curi L, Orihuela S. Chronic pain treatment with cannabidiol in kidney transplant patients in uruguay. *Transplant Proc.* (2018) 50:461–4. doi: 10.1016/j.transproceed.2017.12.042
81. Tartaglia N, Bonn-Miller M, Hagerman R. Treatment of Fragile X syndrome with cannabidiol: a case series study and brief review of the literature. *Cannabis cannabinoid Res.* (2019) 4:3–9. doi: 10.1089/can.2018.0053
82. Heussler H, Cohen J, Silove N, Tich N, Bonn-Miller MO, Du W, et al. A phase 1/2, open-label assessment of the safety, tolerability, and efficacy of transdermal cannabidiol (ZYN002) for the treatment of pediatric fragile X syndrome. *J Neurodev Disord.* (2019) 11:16. doi: 10.1186/s11689-019-9277-x
83. Bergamaschi MM, Queiroz RHC, Chagas MHN, de Oliveira DCG, De Martinis BS, Kapczinski F, et al. Cannabidiol reduces the anxiety induced by simulated public speaking in treatment-naïve social phobia patients. *Neuropsychopharmacol.* (2011) 36:1219–26. doi: 10.1038/npp.2011.6
84. Chagas MHN, Eckeli AL, Zuardi AW, Pena-Pereira MA, Sobreira-Neto MA, Sobreira ET, et al. Cannabidiol can improve complex sleep-related behaviours associated with rapid eye movement sleep behaviour disorder in Parkinson's disease patients: a case series. *J Clin Pharm Ther.* (2014) 39:564–6. doi: 10.1111/jcpt.12179
85. Verma AK, Khan E, Mishra SK, Mishra A, Charlet-Berguerand N, Kumar A. Curcumin regulates the r(CGG)(exp) RNA hairpin structure and ameliorate defects in Fragile X-associated tremor ataxia syndrome. *Front Neurosci.* (2020) 14:295. doi: 10.3389/fnins.2020.00295
86. Shokri-Mashhadi N, Bagherniya M, Askari G, Sathyapalan T, Sahebkar A. A Systematic review of the clinical use of curcumin for the treatment of osteoarthritis. *Adv Exp Med Biol.* (2021) 1291:265–82. doi: 10.1007/978-3-030-56153-6_16
87. Napoli E, Flores A, Mansuri Y, Hagerman RJ, Giulivi C. Sulforaphane improves mitochondrial metabolism in fibroblasts from patients with fragile X-associated tremor and ataxia syndrome. *Neurobiol Dis.* (2021) 157:105427. doi: 10.1016/j.nbd.2021.105427
88. Patte-Mensah C, Meyer L, Taleb O, Mensah-Nyagan AG. Potential role of allopregnanolone for a safe and effective therapy of neuropathic pain. *Prog Neurobiol.* (2014) 113:70–8. doi: 10.1016/j.pneurobio.2013.07.004
89. Nair AS, Diwan S. Allopregnanolone: A neurosteroid for managing acute and chronic pain conditions. *Saudi J Anaesth.* (2019) 13:264–6. doi: 10.4103/sja.SJA_830_18
90. Meltzer-Brody S, Kanes SJ. Allopregnanolone in postpartum depression: Role in pathophysiology and treatment. *Neurobiol Stress.* (2020) 12:100212. doi: 10.1016/j.ynstr.2020.100212
91. Wang JY, Trivedi AM, Carrillo NR, Yang J, Schneider A, Giulivi C, et al. Open-label allopregnanolone treatment of men with fragile X-associated tremor/ataxia syndrome. *Neurother J Am Soc Exp Neurother.* (2017) 14:1073–83. doi: 10.1007/s13311-017-0555-6
92. Meeus M, Nijs J, Hermans L, Goubert D, Calders P. The role of mitochondrial dysfunctions due to oxidative and nitrosative stress in the chronic pain or chronic fatigue syndromes and fibromyalgia patients: peripheral and central mechanisms as therapeutic targets? *Expert Opin Ther Targets.* (2013) 17:1081–9. doi: 10.1517/14728222.2013.818657
93. Grimm A, Schmitt K, Lang UE, Mensah-Nyagan AG, Eckert A. Improvement of neuronal bioenergetics by neurosteroids: implications for age-related neurodegenerative disorders. *Biochim Biophys Acta.* (2014) 1842:2427–38. doi: 10.1016/j.bbadis.2014.09.013
94. Turk DC, Meichenbaum D. A cognitive-behavioral approach to pain management. In: *The Evolution of Cognitive Behavior Therapy: A Personal and Professional Journey with Don Meichenbaum.* (2017). p. 132–54.
95. Finan PH, Goodin BR, Smith MT. The association of sleep and pain: an update and a path forward. *J pain.* (2013) 14:1539–52. doi: 10.1016/j.jpain.2013.08.007
96. Sielski R, Rief W, Glombiewski JA. Efficacy of Biofeedback in Chronic back Pain: a Meta-Analysis. *Int J Behav Med.* (2017) 24:25–41. doi: 10.1007/s12529-016-9572-9
97. Hilton L, Hempel S, Ewing BA, Apaydin E, Xenakis L, Newberry S, et al. Mindfulness Meditation for Chronic Pain: Systematic Review and Meta-analysis. *Ann Behav Med.* (2017) 51:199–213. doi: 10.1007/s12160-016-9844-2
98. Manheimer E, White A, Berman B, Forys K, Ernst E. Meta-analysis: acupuncture for low back pain. *Ann Intern Med.* (2005) 142:651–63. doi: 10.7326/0003-4819-142-8-200504190-00014
99. Vickers AJ, Rees RW, Zollman CE, McCarney R, Smith CM, Ellis N, et al. Acupuncture for chronic headache in primary care: large, pragmatic, randomised trial. *BMJ.* (2004) 328:744. doi: 10.1136/bmj.38029.421863.EB

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